The role of dietary choline on atherosclerosis development

by

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Abstract

Choline, as an essential nutrient, is needed for a variety of biological processes such as phospholipid synthesis, cell-membrane signaling, lipoprotein secretion, acetylcholine biosynthesis, and one-carbon metabolism. In the North American population diet the two most common forms of choline in foods are phosphatidylcholine (PC) and free-choline (FC). The recommended adequate intake (AI) is 550 and 425 mg/d for men and women, respectively. However, it has been reported that choline intake is below the AI in various populations and it has been recommended to increase the consumption of foods that are rich in choline. During the last decade epidemiological studies have suggested that consumption of choline rich foods (specifically PC from animal source) might increase cardiovascular disease (CVD) risk. Excess dietary choline is metabolized by gut microorganisms to trimethylamine (TMA) and then is further oxidized in the liver to form trimethylamine N-oxide (TMAO). It has been proposed that TMAO might enhance atherosclerosis development and be a biomarker for CVD risk. Therefore, the objective of this thesis was to understand the role of dietary choline supplementation on atherosclerosis development.

We conducted a series of feeding trials to investigate whether the form of dietary choline, free choline or PC supplementation influences atherosclerosis development in atherogenic mouse models. It was observed that in *Ldlr*$^{-/-}$ mice, dietary supplementation with choline or TMAO increased plasma TMAO levels by 1.6- and 4-fold, respectively after 8 wk. Meanwhile, after 16 wk there was an increase of 2-fold at TMAO supplementation. In *Apoe*$^{-/-}$ mice, plasma TMAO levels were significantly (p<0.05) different only between choline and betaine supplemented for 12 wk. However, following the dietary intervention for 28 wk in this mouse model, TMAO levels were significantly (p<0.05) increased in choline and TMAO groups compared to controls, 1.8 and

1.5-fold respectively. These dietary interventions did not alter atherosclerosis or plasma cholesterol levels in either mouse model.

Surprisingly, in *Ldlr*$^{-/-}$ mice PC supplementation reduced atherosclerotic lesions (p<0.05) while having 2-fold higher plasma TMAO levels compared with both control and choline supplemented diets (p<0.05). At fasting state, PC supplementation decreased (p<0.05) plasma VLDL-C and APOB48, and increased (p<0.05) plasma HDL-C. However, VLDL secretion was not affected by dietary treatment. In spleen and peripheral blood immune cell phenotypes there were no differences in the proportion of T and B cells subsets and macrophages along with the activation markers that they express including ICAM-1. Nevertheless, we observed lower (p<0.05) levels of circulating pro-atherogenic chemokines in the PC supplemented group. This study suggests that increased dietary PC intake does not induce a pro-atherogenic phenotype.

In parallel a randomized controlled crossover in healthy men was performed. We examined the effect of standardized breakfasts containing different dietary amounts, forms, and sources of choline on postprandial choline and TMAO metabolism in healthy men. The high choline meals (~360 mg of choline) provided to the participants did not impact postprandial TMA, TMAO, choline response. However, plasma TMAO levels were increased (p<0.05) after 0.5 and 1 h of consuming a high free-choline breakfast compared to high PC breakfasts.

Overall, it was concluded that dietary choline supplementation did not enhance atherosclerosis despite increasing plasma TMAO levels. Surprisingly, dietary PC supplementation reduced atherosclerotic development. In healthy men, it was showed that a single high choline meal did not alter the postprandial TMAO response.

Preface

This thesis is original work by Paulina Aldana Hernandez. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta's Institutional Animal Care Committee and is listed as *'Dietary determinants of metabolic disorders'* with protocol number AUP00000175, July 2009. The research project involving humans, of which this thesis is a part, received research ethics approval from the University of Alberta Health Research Ethics Biomedical Panel *'Choline Breakfast Study'* Pro00068657, December 2016. The contributions made by the candidate, Paulina Aldana Hernandez, and the co-authors of these studies, are described below.

Chapter 2 of this thesis has been published as Aldana-Hernández, P., Leonard, K.-A., Zhao, Y.-Y., Curtis, J. M., Field, C. J., & Jacobs, R. L. (2020). Dietary Choline or Trimethylamine N-oxide Supplementation Does Not Influence Atherosclerosis Development in *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ Male Mice. *The Journal of Nutrition*, *10*, 1–7. PAH was responsible for conducting the research, analyzing and performing statistical analyses of the data, and preparing the manuscripts. JMC, RLJ, and CJF: designed the research. PAH and KAL conducted the research. PAH, KAL, and YYZ analyzed the data. PAH performed the statistical analyses. PAH and RLJ wrote the paper. RLJ has primary responsibility for the final content.

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research. PAH, JAB, JNV, KAL, YYZ, and SG analyzed the data. PAH performed the statistical analyses. PAH and RLJ wrote the paper. RLJ has primary responsibility for the final content.

Dedication

In memory of my father, Gerardo Aldana Lara. Thank you for always spoiling and providing me the best education to reach my full potential.

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List of Abbreviations

AI - adequate intake

APOA1 - apolipoprotein A1

APOB - apolipoprotein B

APOE - apolipoprotein E

Apoe$^{-/-}$ - Apolipoprotein E knockout

BADH - betaine aldehyde dehydrogenase

BAT - brown adipose tissue

BHMT - betaine homocysteine methyltransferase

BMI - body mass index

CD36 - cluster of differentiation 36

CETP - cholesterol ester transfer protein

ChAT - choline acyltransferase

CHD - coronary heart disease

CHDH - choline dehydrogenase

CK - choline kinase

CON - control

CS - choline-supplemented

CT - CTP:phosphocholine cytidylyltransferase

CTP - choline phosphotransferase

CVA - cerebrovascular accident

CVD - cardiovascular disease

CVE - cardiovascular event (composite of myocardial infarction, stroke, and death from cardiovascular causes)

DAG - diacylglycerol

DASH - Dietary Approaches to Stop Hypertension

DHA - docosahexaenoic acid

EBS - *Apoe*$^{-/-}$ betaine-supplemented

EC - *Apoe*$^{-/-}$ control

ECM - extracellular matrix

ECS - *Apoe*$^{-/-}$ choline-supplemented

EPA - eicosapentaenoic acid

ER - endoplasmic reticulum

ETS - *Apoe*$^{-/-}$ TMAO-supplemented

FC - free-choline

FFA - free fatty acids

FMO - flavin-containing monooxygenase

GDE - glycerophosphodiester phosphodiesterase

GDPD - glycerophosphocholine phosphodiesterase

GI - low glycemic index

GPC - glycerophosphocholine

HC - high choline

HC-HTMAO - high choline in the high TMAO baseline group

HC-LTMAO - high choline in the low TMAO baseline group

HDL - high-density lipoprotein

HDL-C - high-density lipoprotein-cholesterol

HFC - high FC

HFC-HTMAO - high free-choline in the high TMAO baseline group

HFC-LTMAO - high free-choline in the low TMAO baseline group

HFD - high-fat diet

HILIC - hydrophilic interaction chromatography

HNRU - Human Nutrition Research Unit

HPC - high PC

HPC-HTMAO - high PC in the high TMAO baseline group

HPC-LTMAO - high PC in the low TMAO baseline group

HPCM - high PC meal

HPCM-HTMAO - high PC meal in the high TMAO baseline group

HPCM-LTMAO - high PC meal in the low TMAO baseline group

HPCS - high PC from supplement

HPCS-HTMAO - high PC from supplement in the high TMAO baseline group

HPCS-LTMAO - high PC from supplement in the low TMAO baseline group

ICAM-1 - Intercellular Adhesion Molecule 1

IV - intravenous

KC - keratinocyte chemoattractant

LBS - *Ldlr*$^{-/-}$ betaine-supplemented

LCM - low choline

LC - *Ldlr*$^{-/-}$ control

LCAT - lecithin-cholesterol acyltransferase

LC-HTMAO - Low choline in the high TMAO baseline group

LC-LTMAO - Low choline in the low TMAO baseline group

LC-MS/MS - liquid chromatography–tandem mass spectrometry

LCS - *Ldlr*$^{-/-}$ choline-supplemented

LDL - low-density lipoprotein

LDLR - LDL receptor

Ldlr$^{-/-}$ - low-density lipoprotein receptor knockout

LPC - lysophosphatidylcholine

LPCATs - lysophosphatidylcholine acyltransferases

LPL - lysophospholipase

LTS - *Ldlr*$^{-/-}$ TMAO-supplemented

MA - meta-analysis

MCP1 - macrophage chemo attractant protein 1

MD - mean differences

MI - myocardial infarction

MIP-1α - macrophage inflammatory protein-1α

MSR1 - macrophage scavenger receptors type 1

M-THF - methyl-tetrahydrofolate

MUFA - monounsaturated fatty acid

OCM - one-carbon metabolism

PC - phosphatidylcholine

PCS - PC-supplemented

PE - phosphatidylethanolamine

PEMT - phosphatidylethanolamine N-methyltransferase

PhC - phosphocholine

PLA2 - phospholipase A2

PLB - phospholipase B

PLC - phospholipases C

PLD1 - phospholipase D1

PUFAs - polyunsaturated fats

RChT - reverse cholesterol transport

RCT - randomized controlled trial

RR - relative risk

SAH - S-adenosylhomocysteine

SAM - S-adenosylmethionine

SFAs - saturated fats

SM - sphingomyelin

SMC - smooth muscle cell

SMs - sphingomyelinase

SR - systematic review

SRREs - summary relative risk estimates

TG - triacylglycerol

THF - tetrahydrofolate

TLR-4 - Toll-like receptor 4

TMA - trimethylamine

TMAO - trimethylamine N-oxide

USDA - United States Department of Agriculture

VLDL - very low-density lipoprotein

VLDL-C - very low-density lipoprotein-cholesterol

WHO - World Health Organization

WMD - weighted mean difference

List of Appendices

Chapter 1: Introduction and literature review

1.1 Choline, an essential nutrient

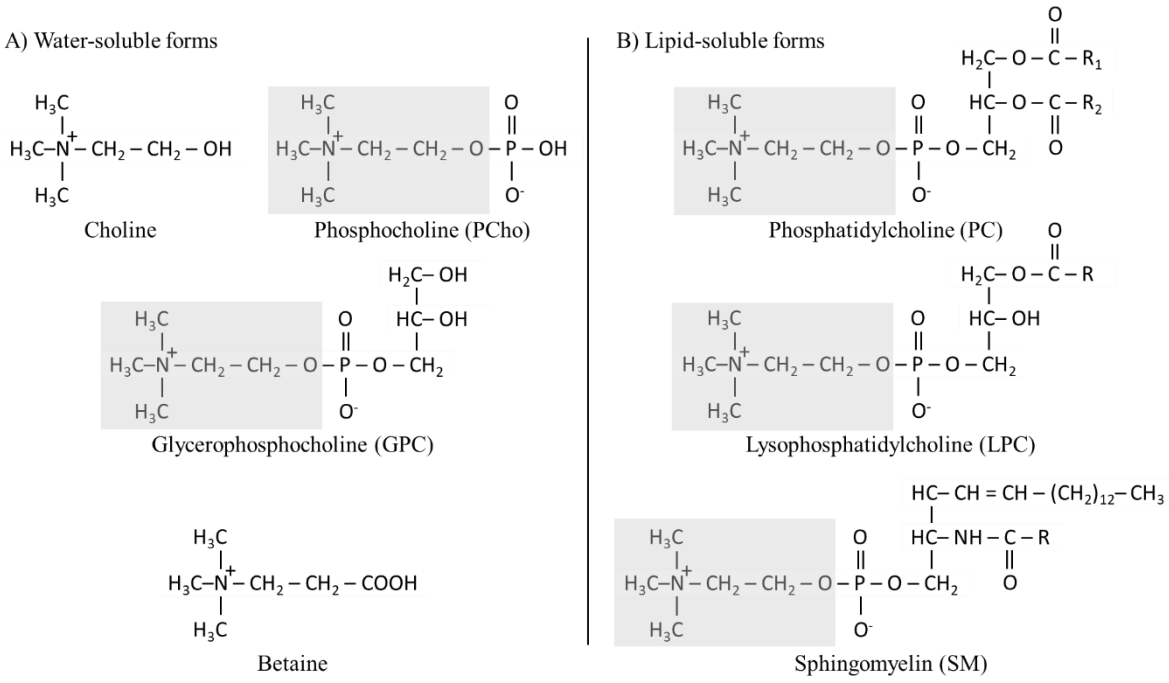
In 1998, when The Institute of Medicine updated the Dietary Reference Intakes for B vitamins, choline officially became an essential nutrient for humans (Institute of Medicine 1998). Choline is required for a variety of biological processes such as cell membrane formation via phospholipid synthesis, lipoprotein secretion, acetylcholine biosynthesis, and as a methyl donor group when it is oxidized to betaine. Choline can be obtained through *de novo* biosynthesis and diet (Zeisel and da Costa 2009). The *de novo* biosynthesis of choline takes place mainly in the liver by three consecutive methylations of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) (Vance 2014) catalyzed by the enzyme, phosphatidylethanolamine N-methyltransferase (PEMT). However, the amount of choline synthesized *de novo* is not always sufficient, thus the adequate intake (AI) was set at 550 and 425 mg/d for men and women, respectively (Institute of Medicine 1998).

1.1.1 Moieties and dietary sources of choline

Choline is present in an extensive variety of foods (Zeisel 2006). It is found in meat (animal liver), eggs, cereals, milk and dairy products, and in vegetables, such as soybeans and spinach (Lewis, Subhan, et al. 2014; Zeisel et al. 2003). Typically, the choline database from the United States Department of Agriculture (USDA) is used to determine the total choline and choline moieties intake in the population (Patterson et al. 2008a). Recently, our research group developed the Alberta choline food database that includes a greater range of meat types and pulse products than the USDA choline database (Lewis 2016; Lewis, Kosik, et al. 2014). This database includes approximately 3,800 items commonly consumed in North America, which can be used to estimate the choline intake in a widespread population.

Our food supply contains both water-soluble and lipid-soluble forms of choline (*Figure 1.1*). The water-soluble forms include free-choline (FC), phosphocholine (PhC), glycerophosphocholine (GPC), and betaine (oxidized choline) (Zeisel and Blusztajn 1994). The lipid-soluble forms are phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SM) (Zeisel and Blusztajn 1994).

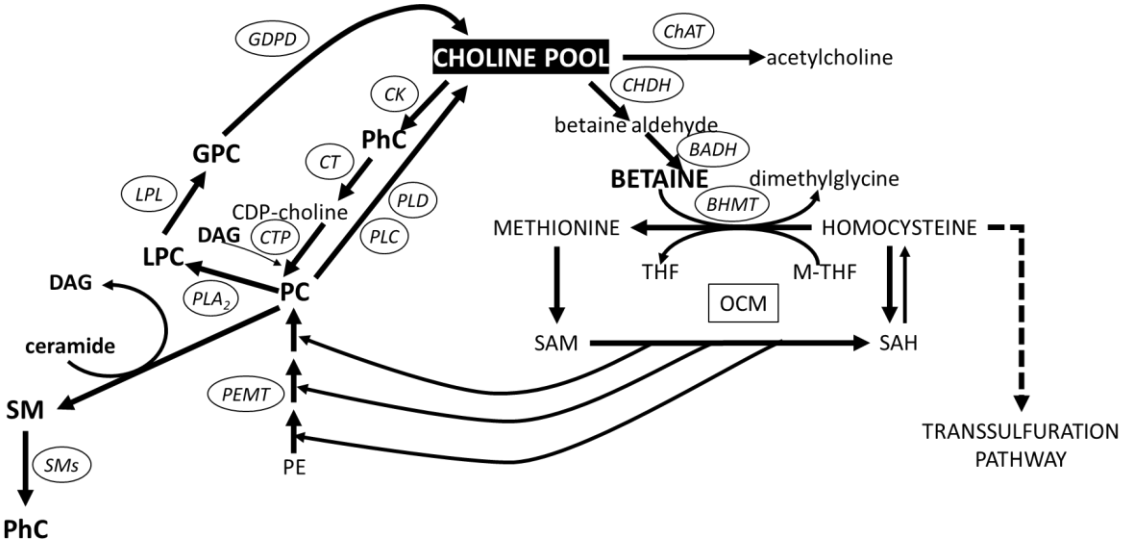
Figure 1.1 Structures of dietary forms of choline



1.1.2 Biosynthesis of choline

Endogenously, free-choline is obtained through the lysis and biosynthesis of PC (*Figure 1.2*). PC is synthesized through three consecutive methylations of phosphatidylethanolamine (PE), catalyzed by the enzyme PEMT, which is mainly found in the liver (Bremer and Greenberg 1960; van der Veen, Kennelly, et al. 2017). The methyl-groups used by the PEMT come from one-carbon metabolism (OCM). It is estimated that ~30% of the PC biosynthesized in the liver comes from PEMT (Vance 2014; van der Veen, Kennelly, et al. 2017). The primary PC biosynthesis pathway in the liver (and all other tissues) is called the CDP-choline (or Kennedy pathway) where choline is the substrate (Vance 2014; van der Veen, Kennelly, et al. 2017). Inside the cell, choline is phosphorylated to PhC via choline kinase (CK) by ATP. Later, CTP and PhC are converted to CDP-choline by the action of the enzyme CTP:phosphocholine cytidylyltransferase (CT); this is the rate-limiting reaction for this pathway. Finally, in the endoplasmic reticulum (ER), choline phosphotransferase (CTP) transfers PhC from CDP-choline to diacylglycerol (DAG) and generates PC (Vance and Vance 1990). In order to compensate the poor choline pool, PC could be hydrolyzed by the action of the phospholipases C (PLC)and D (PLD) (Liscovitch et al. 1993; Nishio et al. 1992).

Figure 1.2 Choline-metabolites biosynthesis



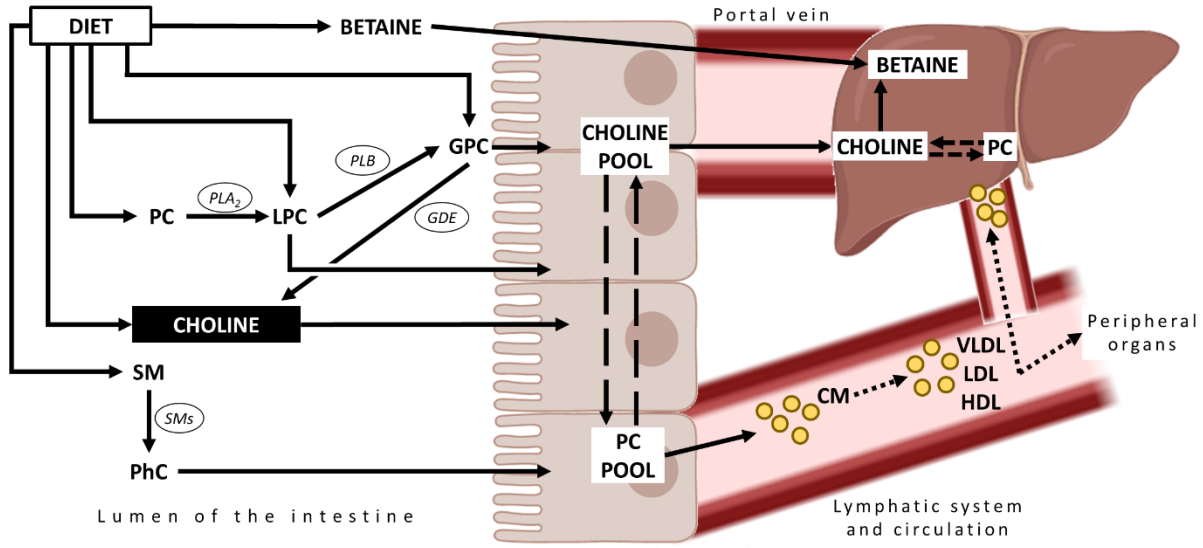
Abbreviations: betaine aldehyde dehydrogenase, BADH; betaine homocysteine methyltransferase, BHMT; choline acyltransferase, ChAT; choline dehydrogenase, CHDH; choline kinase, CK; CTP:phosphocholine cytidylyltransferase, CT; choline phosphotransferase, CTP; diacylglycerol, DAG; glycerophosphocholine phosphodiesterase, GDPD; glycerophosphocholine, GPC; lysophosphatidylcholine, LPC; lysophospholipase, LPL; methyl-tetrahydrofolate, M-THF; one-carbon metabolism, OCM; phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylcholine-N-methyltransferase, PEMT; phosphocholine, PhC; phospholipase A_2, PLA_2; phospholipase C, PLC; phospholipase D, PLD; S-adenosylhomocysteine, SAH; S-adenosylmethionine, SAM; sphingomyelin, SM; sphingomyelinase, SMs; tetrahydrofolate, THF.

1.1.3 Absorption and metabolism of choline

Choline is widely present in food as both water-and lipid-soluble forms, thus are metabolized by the small intestine by different mechanisms. FC, which represents ~40% of dietary choline, is absorbed by specific transporters (Sheard and Zeisel 1986; Zeisel, Wishnok, and Blusztajn 1983). PC is hydrolyzed to LPC and free fatty acids (FFA) by the pancreatic enzymes phospholipase A2 (Arnesjö et al. 1969; Borgström 1993; Nilsson and Duan 2019). GPC is absorbed intact but can be hydrolyzed to FC by glycerophosphodiester phosphodiesterase in the intestinal mucosal cells (Nilsson and Duan 2019). Meanwhile, on the surface of the microvilli, the alkaline sphingomyelinase hydrolyzes SM to ceramide and PhC (Nilsson and Duan 2019). After being absorbed, water-soluble cholines are mainly transported to the liver via portal vein. Alternatively, the lipid-soluble cholines are packed into chylomicrons and transported into the lymphatic system (Zeisel 1981). Betaine from diet is absorbed by sodium-dependent transporter in the duodenum and enters circulation via the portal vein (Kettunen et al. 2001).

In liver and intestine, the PC pool is maintained from FC *via* the CDP-choline pathway (Figure 1.2) and from LPC *via* lysophosphatidylcholine acyltransferases (LPCATs). LPCAT catalyzes the addition of a FA into the *sn-2* position of the LPC to form PC (Wang and Tontonoz 2019). The synthesis of PC is required for normal packaging and release for TG-rich lipoproteins (van der Veen, Kennelly, et al. 2017). As well, FC contributes as methyl-donor group when is oxidized to betaine by the betaine homocysteine methyltransferase (BHMT) (Figure 1.3).

Figure 1.3 Absorption of choline metabolites

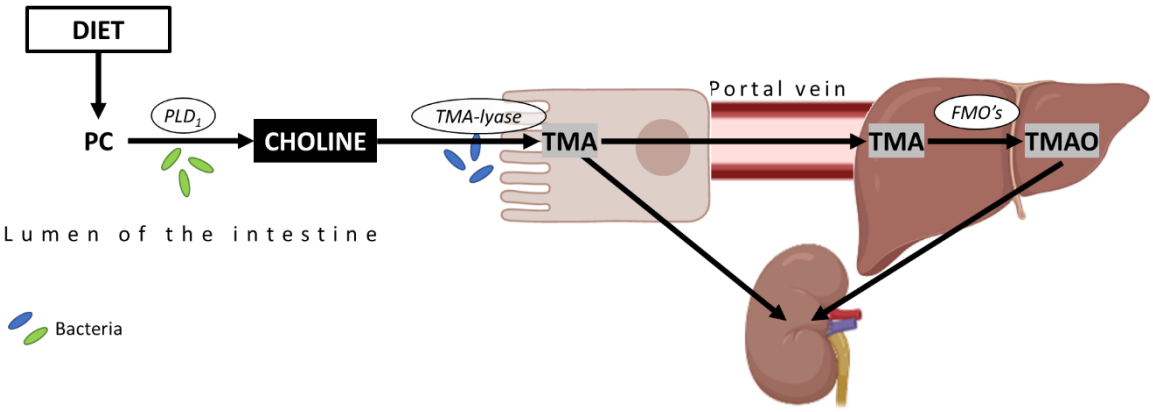


Abbreviations: glycerophosphocholine, GPC; glycerophosphodiester phosphodiesterase, GDE; lysophosphatidylcholine, LPC; phosphatidylcholine, PC; phosphocholine, PhC; phospholipase A_2, PLA_2; phospholipase B, PLB; sphingomyelin, SM; sphingomyelinase, SMs

1.1.4 Choline and trimethylamine-N-oxide production

Recently, it was reported that the bacterial enzyme phospholipase D_1 (PLD_1) hydrolyzes PC to FC (Chittim, Martínez del Campo, and Balskus 2019). Excess FC that reaches the large intestine is metabolized by *TMA-lyases* from the gut microbiota to trimethylamine (TMA). TMA is absorbed by the host and oxidized in the liver to trimethylamine-N-oxide (TMAO) by a group of enzymes named flavin-containing monooxygenases (FMO's) (Zeisel et al. 1983). Both TMA and TMAO are excreted from the body via the urine.

Figure 1.4 Absorption, metabolism, and secretion of TMA and TMAO

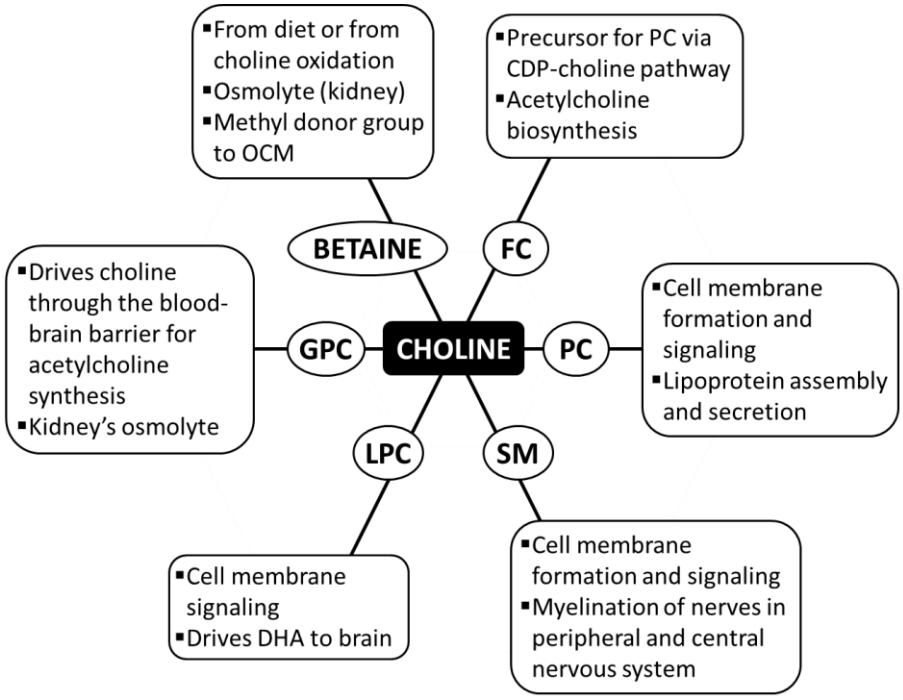


Abbreviations: flavin-containing monooxygenases, FMO's; phosphatidylcholine, PC; phospholipase D_1, PLD_1; trimethylamine, TMA; trimethylamine N-oxide, TMAO

1.1.5 Physiological roles of choline

Choline plays an important role in a variety of biological processes summarized in Figure 1.5.

Figure 1.5 Biological roles of choline



Abbreviations: free-choline, FC; glycerophosphocholine, GPC; lysophosphatidylcholine, LPC; phosphatidylcholine, PC; sphingomyelin, SM

1.1.5.1 Cell membrane formation and signaling

Phospholipids are the main component of cell membranes by forming a bilayer structure. PC represents ~50% of these phospholipids (van Meer, Voelker, and Feigenson 2008). Another choline-molecule present in the membrane is SM (van Meer et al. 2008). Both PC and SM play a role in intracellular and extracellular signaling, crucial for processes like cell differentiation and growth, stimulation of phagocytosis, enzyme activity regulation, and apoptosis (Zeisel and Blusztajn 1994). Impairment of either PC synthesis pathway alter membrane resulting in lipid bilayer stress, which may cause ER stress (Fu et al. 2011; Gao et al. 2015; Li et al. 2006; Niebergall et al. 2011; Op den Kamp 1979).

1.1.5.2 Lipoprotein assembly and secretion

PC is required for the assembly of lipoproteins that transport lipids; obtained exogenously (from diet) in the enterocytes and endogenously (de novo synthesis or from adipose) in the liver. Approximately 80% of the lipoproteins in circulation come from the liver and the rest is formed in the intestine (Vance and Vance 1990). The assembly of very low-density lipoprotein (VLDL) in the liver requires the lipidation (TG and PC) of apolipoprotein B (apoB) in the rough ER and is secreted through the Golgi apparatus (Vance and Vance 1990). Impairment of PC synthesis impacts TG secretion from the liver. For example, hepatic-specific CTα knock out mice have reduced PC synthesis, impaired VLDL secretion, and elevated hepatic TG stores (Jacobs et al. 2004, 2008). Similarly, hepatocytes from *Pemt*$^{-/-}$ mice secreted 50% less TG and cholesterol leading to a reduction in circulating VLDL and LDL fraction (Noga, Zhao, and Vance 2002). The reduction in VLDL secretion leads to an accumulation of hepatic TG when *Pemt*$^{-/-}$ mice are fed a HFD (Noga and Vance 2003a; Al Rajabi et al. 2014; van der Veen, Lingrell, et al. 2017).

1.1.5.3 Choline/betaine as methyl-donors

Betaine comes from diet and choline oxidation, mainly in the liver and kidney. In the mitochondria, choline is irreversible oxidized first to betaine aldehyde by choline dehydrogenase (CHDH) and then to betaine by betaine aldehyde dehydrogenase (BADH) (Day and Kempson 2016). The OCM occurs nearly in all cells and impacts many physiological functions such as cellular biosynthesis of purines, regulation of redox status, homeostasis of amino acids, and epigenetic methylation (Ducker and Rabinowitz 2017) by using methyl-donor groups like folate and betaine (Figure 1.2). To form methionine, betaine donates a methyl group to homocysteine through the action of BHMT (Day and Kempson 2016). Methionine, not used for protein synthesis, is converted to S-adenosylmethionine (SAM), which is used to methylate DNA and proteins, synthesize PC, hormones, and other bioactive methylated molecules, such as creatine (Tahiliani et al. 2009; Taunton, Hassig, and Schreiber 1996; Watkins, Zhu, and Zeisel 2003).

1.1.5.4 Other functions of choline metabolites

Betaine and GPC play a role as osmolytes in renal medullary cells. These molecules are compatible with solutes to maintain cell volume and electrolyte levels versus variable hypertonicity (Burg 1996). SM is necessary for the myelination of neurons in the central and peripheral nervous system (Wattenberg 2019). Acetylcholine is biosynthesized from choline and acetyl-CoA by the enzyme choline acetyltransferase in cholinergic neurons (Blusztajn and Wurtman 1983).

1.1.5.5 The importance of dietary choline intake

As highlighted above, choline plays an important role in number of physiological processes. The development of fatty liver disease is one of the primary consequences of consuming a choline deficient diet in humans (Buchman et al. 1995; Institute of Medicine 1998). A recent study in

healthy adults showed that 77% of the men and 80% of the postmenopausal women developed subclinical organ dysfunction (fatty liver or muscle damage) when deprived of dietary choline (Fischer et al. 2007). However, the damage was quickly reversed when choline was reintroduced into the diet. Fifty percent of premenopausal women did not develop tissue damage (Fischer et al. 2007), which is consistent with estrogen's role as a positive regulator of *de novo* choline synthesis. As well, patients with total parenteral nutrition supplemented with choline shown a reduction of plasma aminotransferase levels (a biomarker for liver damage) compared to controls (Buchman et al. 2001). In mice, impaired PC synthesis reduces VLDL secretion, leading to TG accumulation and liver damage (Jacobs et al. 2004, 2008; Noga and Vance 2003a; Noga et al. 2002). Interestingly, dietary choline supplementation has been shown to improve liver health in mice with impaired hepatic PC synthesis (Jacobs et al. 2010; Niebergall et al. 2011).

1.2 Cardiovascular diseases, a brief description

The World Health Organization (WHO) reports that cardiovascular diseases (CVDs) are the number 1 cause of death worldwide. In 2016 WHO estimated that 17.9 million people died from CVDs (31% of all deaths worldwide), ~ 85% were due to heart attack and stroke (World Health Organization 2017). Current modifiable lifestyle factors have been associated to the risk of CVDs, like tobacco use, low physical activity, overweight and obesity, high blood pressure, abnormal plasma lipid levels, and diet rich in saturated fatty acids and poor in fruit and vegetable (Lovegrove and Hobbs 2016). Due to the increased prevalence of CVDs and the sedentary lifestyle in recent years, several clinical biomarkers have been identified for early detection and prevention of disease progression. Table 1.1 shows a summary of biomarkers at fasting and non-fasting state.

Table 1.1 Biomarkers for CVD risk (Anderson et al. 2016; Miller et al. 2011; Nordestgaard et al. 2016).

Parameter	Fasted	Non-fasted
Blood pressure	>140/90 mm Hg	
TG	≥150 mg/dL (≥1.8 mmol/L)	≥175 mg/dL (≥2 mmol/L)
LDL-Cholesterol	≥115 mg/dL (≥3 mmol/L)	≥115 mg/dL (≥3 mmol/L)
HDL-Cholesterol	≥40 mg/dL (≥1 mmol/L)	≥40 mg/dL (≥1 mmol/L)
Total cholesterol	≥190 mg/dL (≥5 mmol/L)	≥190 mg/dL (≥5 mmol/L)
Remnant cholesterol	≥30 mg/dL (≥0.8 mmol/L)	≥35 mg/dL (≥0.9 mmol/L)
Non-HDL cholesterol	≥145 mg/dL (≥3.8 mmol/L)	≥150 mg/dL (≥3.9 mmol/L)
Glucose	≥ 7.0 mmol/L	
ApoB	≥100 mg/dL	≥100 mg/dL

TG, triacylglycerols; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ApoB, apolipoprotein B

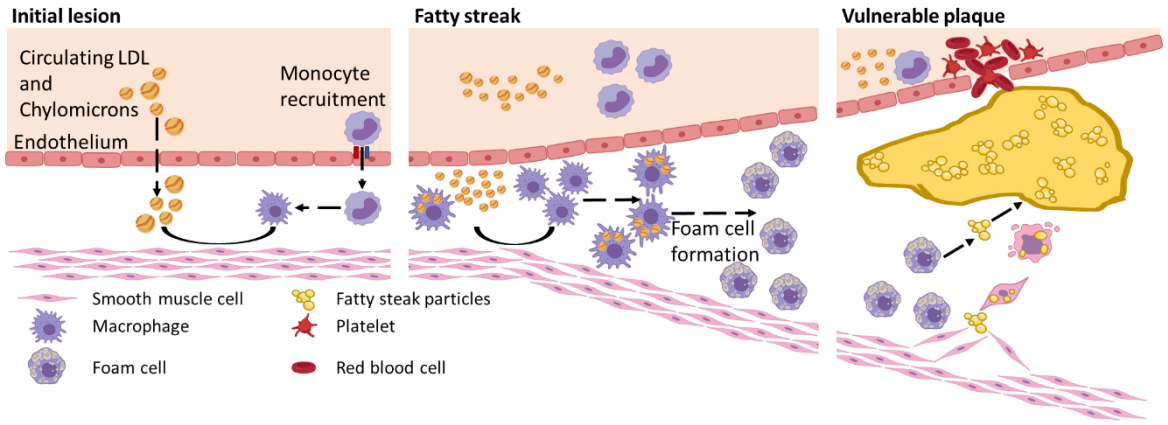
1.2.1 Definition of cardiovascular diseases

CVDs have been defined as a group of disorders of the heart and blood vessels including coronary heart disease (CHD, heart attacks), cerebrovascular disease (stroke), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease, deep vein and thrombosis, pulmonary embolism, and heart failure (World Health Organization 2017). One of the main causes of cerebrovascular disease and CHD is atherosclerosis which slowly progresses from lesion formation in the endothelial walls to narrowing the arteries (Weber and Noels 2011).

1.2.2 Description of atherosclerosis development

Atherosclerosis is characterized by an inflammatory state of the arterial wall led by an accumulation of lipids and a maladaptive inflammatory response (Silvestre-Roig et al. 2014). LDL is the main cholesterol carrier in circulation between the liver and the rest of the peripheral tissues. LDL particles are taken up by endocytosis via LDL receptor (LDLR). To keep cholesterol homeostasis, excess of cholesterol from peripheral tissues is removed through the reverse cholesterol transport process (RChT) by the action of high-density lipoproteins (HDL). HDL transports cholesterol from peripheral tissues back to the liver. Hepatic cholesterol is secreted by the bile system to the intestine (as bile acids or cholesterol), where ~5% is excreted in feces and the rest is reabsorbed. If this homeostatic mechanism is unbalanced the atherosclerosis development could be accelerated (Lewis and Rader 2005; Ohashi et al. 2005).

Figure 1.6 Atherosclerotic plaque formation (Linton et al. 2000; Santhakumar, Battino, and Alvarez-Suarez 2018)



The arterial wall is formed by endothelial cell lining and smooth muscle. During the initial lesion stage, high circulating chylomicrons and LDL-C particles migrate through the endothelium by passive diffusion and are susceptible to modifications such as oxidation, cleavage, and aggregation. Accumulation of ApoB-containing lipoproteins (including chylomicron remnants) stimulates the immune response by the secretion of cytokines and chemokines that activate the expression of intercellular adhesion molecule-1 (ICAM-1) and macrophage chemo attractant protein 1 (MCP1, also known as CCL2). Circulating monocytes are recruited and migrate to the intima where they differentiate into macrophages. In order to clear the lipid aggregation in the arterial wall, activated macrophages ingest normal and modified lipoproteins via macrophage scavenger receptors type 1 (MSR1) and CD36. As the inflammation state continues and impairs the function of macrophages, the RChT process gets impaired. If the progression of the lesion continues macrophages transform into cholesterol-loaded foam cells. The imbalanced function of macrophages leads to both apoptosis and necrosis of the foam cells, provoking the release of the fatty content and a necrotic core is formed. Furthermore, the expansion of the fatty streak leads to the formation of fibrous scar tissue, narrowing of the lumen, thickening the arterial wall, and rupturing of the nearest smooth muscle cells (SMC) layer. The migration of the SMC and the accumulation of stimulated macrophages, endothelial and T cells, forming a fibrous cap around the lipid core contributes to the formation of the extracellular matrix (ECM). The growing ECM increases the plaque rupture risk. Finally, after the rupture of the arterial wall endothelium, platelet aggregation process occurs and leads to the obstruction of the arterial blood flow (Geovanini and Libby 2018; Ilhan 2015; Linton et al. 2000; Moore, Sheedy, and Fisher 2013; Ramji and Davies 2015; Reape and Groot 1999; Reilly et al. 2007; Santhakumar et al. 2018; Tabas and Lichtman 2017; Tousoulis et al. 2016)

1.2.3 Cardiovascular disease and diet, epidemiological evidence

CVDs' causes are multifactorial and most of them might be preventable. Diet modification has shown improvement of CVDs risks and biomarkers (Table 1.2). The 2016 Canadian Society Guidelines for the Management of Dyslipidemia for the prevention of CVD in the adults makes general dietary recommendations and suggestions to reduce CVDs risk based on dietary patterns (Anderson et al. 2016). It encourages moderate caloric intake to achieve and maintain a healthy body weight and reduce CVD risk, by following dietary patterns such as Mediterranean, Portfolio, Dietary Approaches to Stop Hypertension (DASH), which include increasing the consumption of nuts (≥ 30 g/d), legumes, olive oil (≥ 60 mL/d), total fiber (≥ 30 g/d) and whole grains, fruit and vegetables, and consuming a low glycemic load. The Portfolio dietary pattern is recommended to lower LDL-C. This dietary pattern is characterized by including high intake of nuts, soy protein, plant sterols, viscous soluble fiber (≥ 30, 30, 2, and 10 g/d, respectively). As well for LDL-C lowering, dietary patterns rich in pulses (≥130 g/d) low glycemic index (GI), and DASH dietary patterns are suggested (Anderson et al. 2016). The guidelines also suggest that saturated fats (SFAs) should be limited to <9% of total calories and should be replaced with polyunsaturated fats (PUFAs), making an emphasis on n-3/n-6 PUFAs sources (canola and soybean oils). Other recommendations include the suggestion of replacing SFAs with MUFAs from plant sources (like canola or olive oil) and increasing high-quality sources of carbohydrates (like whole grains and low GI) (Anderson et al. 2016).

Emerging prospective cohorts and dietary trials have investigated a specific type of food (fish, egg, dairy, nuts, and fruit and vegetable intake) on CVD risk. It has been demonstrated that the consumption of vegetable oils that contain n-3 PUFA reduces circulating LDL-C and CHD incidence as compared to saturated fat (Alhassan et al. 2017; Aung et al. 2018; Jayedi et al. 2018;

Manson et al. 2019; Qin et al. 2018; Ursoniu et al. 2017). Recent studies have shown a small association of high consumption of red and processed meat with increase CVD risk, even though there was a high heterogeneity in these studies (Guasch-Ferré et al. 2019; Vernooij et al. 2019). However, consuming ≥0.5 serving of meat per day did not affect CVD biomarkers (O'Connor, Kim, and Campbell 2017). Eggs are a good source of cholesterol, protein, unsaturated FA, and phospholipids. In the last decade plenty evidence have shown that egg consumption is not associated with CVD risk (Alexander et al. 2016; Drouin-Chartier et al. 2020; Richard, Cristall, et al. 2017). Studies have shown that moderate dairy (a good source of fat and choline) consumption, especially fermented dairy products might provide a beneficial effect on CVDs (Benatar, Sidhu, and Stewart 2013; Companys et al. 2020; Jakobsen et al. 2021). Including nuts (rich in unsaturated FA and soluble fiber) and whole grain in the diet have exhibited a reduction of CVD biomarkers and incidence (Aune, NaNa Keum, et al. 2016; Aune, Nana Keum, et al. 2016; G.-C. Chen et al. 2016; Del Gobbo et al. 2015; Ho et al. 2016). Overall, there is strong evidence that promoting a balanced nutrition pattern reduces CVDs risk.

Table 1.2 Characteristics of studies of dietary interventions on CVD outcomes

Type of study	Dietary intervention	N	Population details	CVDs biomarkers outcomes	Year	Reference
			Type of food			
Red meat						
MA of 70 prospective cohort studies	Red meat intake (>50 g per serving)	6,035,051 participants	Adults with or without cardiometabolic condition	Small decrease in risk for all-cause mortality with low intake of red or processed meat No association with risk of stroke	2019	(Vernooij et al. 2019)
SR-MA of 36 RCTs	Diets with red meat vs diet replacing red meat	1,803 participants (interventions ≥2 weeks)	Adults aged ≥18 years old	Small decrease in TG WMD= 0.065 mmol/L (95% CI: 0.000–0.129) No difference in LDL-C, HDL-C apoA1 and B, or blood pressure	2019	(Guasch-Ferré et al. 2019)
SR-MA of 24 RCTs	Effects of consumption of ≥0.5 or <0.5 servings of red meat/d on CVD risk factors	1,074 participants	Adults aged ≥19 years old with or without cardiometabolic condition	No differences in lipoprotein profiles or blood pressure	2017	(O'Connor et al. 2017)
Fish and PUFA						
SR-MA of 14 RCTs	Consumption of fish oil (20 to 500 g, once a week to daily)	1,378	Healthy people, overweight/obese, and metabolic syndrome patients.	↓ TG (-0.11 mmol/L; 95% CI -0.18 to -0.04; p=0.002) and ↑ HDL-C (0.06 mmol/L, 95% CI 0.02 to-0.11; p=0.008)	2017	(Alhassan et al. 2017)
SR-MA of 7 RCTs with a parallel or crossover design	Krill oil supplementation at least 4 weeks. 500-mg capsules or softgels of krill oil (dosages 500-4000 mg/d)	662 (427 krill oil group and 235 control group)	Healthy individuals and dyslipidemic individuals	↓ TG (-14.03 mg/dL; 95%CI: -21.38 to -6.67; p<0.001), ↓LDL-C (-15.52 mg/dL; 95% CI: -28.43 to -2.61; p=0.018), and ↑ HDL-C (6.65mg/dL; 95%CI: 2.30 to 10.99; p=0.003)	2017	(Ursoniu et al. 2017)
MA of 5 prospective cohort studies	Fatty and lean intake	123,681 (3,066 cases)	Healthy individuals	↓ ischemic stroke High fatty fish intake RR=0.88 (95%CI: 0.74–1.04)	2018	(Qin et al. 2018)

				High lean fish intake RR=0.81 ((95% CI, 0.67–0.99)		
MA of 14 prospective cohort studies	Effect of fish intake (20 g/d) on all cause and CVD mortality	911,348 participants (75,451 incident of death)	General adult population	inversely related to CVD mortality RR=0.96(95%CI: 0.94-0.98)	2018	(Jayedi et al. 2018)
MA of 10 RCTs	n-3 PUFA supplementation vs control	77,917 participants	Patients (mean 64 years-old) with prior history of CHD, stroke, or diabetes	Not effect on lowering CVD risk	2018	(Aung et al. 2018)
RCT	of vitamin D3 (2000 IU/d) and marine Ω−3 PUFA (1 g/d fish-oil with 840 mg of n−3 PUFA, including 460 mg of EPA and 380 mg of DHA)	25,871 individuals 5.3 years of follow-up	Patients in primary prevention of CVD and cancer >50 years old	Not effect on lowering CVD risk Major CVE in 386 at the Ω−3 group and 419 at placebo group (HR, 0.92; 95%CI: 0.80-1.06; p=0.24)	2019	(Manson et al. 2019)
Vegetable oils and nuts						
MA of 60 RCTs	Interventions >13 d and constant cholesterol intake	159 diet data points and included 1672 volunteers	Healthy adults >17 years old	Risk is reduced when *trans* FA and SFA are replaced with *cis* MUFA by ↑ total:HDL-C ratio	2003	(Mensink et al. 2003)
MA of 32 prospective cohort studies	Consumption of combination of MUFA (plant and animal), olive oil, oleic acid, and MUFA:SFA ratio	841,211 participants and 3.7 to 30 years of follow-up	Healthy adults and patients with history of CVDs > 20 years old	↓risk for: all-cause mortality RR=0.89(95% CI: 0.83-0.96; p=0.001), CV mortality RR=0.88 (95% CI: 0.80-0.96; p=0.004), CVE RR=0.91 (95% CI: 0.86-0.96; p=0.001), and stroke RR=0.83 (95% CI: 0.71-0.97; p=0.02).	2014	(Schwingshackl and Hoffmann 2014)
Nuts						
SR-MA of 61 RCTs	Nuts intake (average 28.4 g/d, 3 to 26 weeks)	2,582 participants	Healthy adults aged ≥18 y without known CVD	↓ total cholesterol (24.7 mg/dL; 95%CI: 25.3-24.0), ↓ LDL-C (24.8 mg/dL; 95% CI: 25.5-24.2), ↓ ApoB (23.7 mg/dL; 95% CI:	2015	(Del Gobbo et al. 2015)

				25.2-22.3), and ↓ TG (22.2 mg/dL; 95% CI: 23.8-20.5)		
MA of 20 prospective cohort studies	Nut intake 28 g/d	819,448 individuals	Adult general population	↓risk for: CHD RR=0.71 (95% CI: 0.63–0.80), stroke RR= 0.93 (95% CI: 0.83–1.05), CVD RR=0.79 (95% CI: 0.70–0.88)	2016	(Aune, NaNa Keum, et al. 2016)
Legumes and grains						
MA of 14 RCTs	Diets enriched with 6.5-6.9 g/d of barley β-glucan ≥3weeks	615 participants	Middle aged adults (20-60 years old)	↓ LDL-C (MD=−0.25 mmol/l, 95% CI: −0.30−0.20) and non-HDL-C (MD=−0.31 mmol/l, 95% CI: −0.39−0.23)	2016	(Ho et al. 2016)
MA of 45 prospective cohort studies	Increase in whole grain intake (90 g/day)	ranged from 245,012-705,253	Healthy adults and patients with history of CVDs > 16 years old	↓ risk for: CHD RR=0.81 (95% CI: 0.75-0.87), stroke RR=0.88 (95% CI: 0.75-1.03), CVD RR=0.78 (95% CI: 0.73-0.85), all-cause mortality RR=0.85 (95% CI: 0.80-0.91), and T2DM RR=0.74 (95% CI: 0.56-0.96)	2016	(Aune, Nana Keum, et al. 2016)
MA of 33 prospective cohort studies	Whole-grain or whole-grain products intake (50 g/d)	722,509,966 patients	Healthy adults > 16 years old at baseline	↓ risk for: total mortality RR=0.78 (95% CI: 0.67-0.91), and CVD mortality RR=0.70 (95% CI: 0.61–0.79)	2016	(G.-C. Chen et al. 2016)
Eggs						
MA of 7 prospective cohort studies	Egg intake (high vs low; ~1/d vs <2/weeks)	276,000 and 308,000 participants for CHD and stroke, respectively (6-26 years of follow-up)	Adults >15 years old at baseline	↓ risk for: stroke SRRE=0.88 (95% CI: 0.81–0.97)	2016	(Alexander et al. 2016)
SM-MA of 6 RCTs	High vs low daily egg consumption (12-20 weeks)	547 participants	Adults with metabolic syndrome or T2D, ≥18 years old	6–12 eggs/week intake has no adverse effect on major CVD risk factors	2017	(Richard, Cristall, et al. 2017)
MA of 3 large US prospective cohort studies[a]	Egg weekly intake	215,618 individuals (up to	Adults ≥25 years old	Up to one egg per day is not associated with CVD risk overall	2020	(Drouin-Chartier et al. 2020)

		32 follow-up years)				
Dairy foods						
MA of 20 RCTs	Low vs increasing dairy food intake to ~3.6 serves/day (~26 weeks)	1,677 participants	Healthy adults >19 years old	No effects on cardiometabolic risk factors	2013	(Benatar et al. 2013)
SR-MA of 20 prospective cohort studies	Dairy product intake	1,619,431 individuals	General adult population >20 years old (~13 years of follow-up for events)	High-fat milk (200 g higher intake/ day) positively associated with CHD RR=1.08 (95% CI: 1.00–1.16) and cheese (20 g higher intake/day) inversely associated with CHD RR=0.96 (95% CI: 0.93–0.98) High vs low milk intake inversely associated with ischemic stroke	2021	(Jakobsen et al. 2021)
SR-MA of 20 prospective cohort studies	Fermented dairy food intake	Sample size from 1868 to 409,885 subjects (2–30 years follow-up)	Adult subjects (>18 y old) of all sexes and races with CV risk	Fermented milk ↓ risk of: stroke, CHD, and CVD mortality RR=0.96 (95% CI: 0.94–0.98) Yogurt ↓ risk of: T2D RR=0.73 (95% CI: 0.70–0.76), and metabolic syndrome RR=0.80 (95% CI:0.74–0.87)	2020	(Companys et al. 2020)
SR-MA of 52 RCTs	Probiotic added into dairy matrix (doses ranged from 1 × 104 to 27 × 1010 CFU/d, from 45 days to 24 weeks)	24 to 210 participants	Patients with cardiometabolic disease	↓ of : total cholesterol WMD= -0.46 mmol/L (95% CI:-0.73– -0.19), LDL-C WMD= -0.50 mmol/L (95% CI:-0.77– -0.22), TG WMD= -0.46 mmol/L (95% CI:-0.75– -0.14) ↑ of HDL-C WMD= 0.26 mmol/L (95% CI:0.01– 0.52),	2020	(Companys et al. 2020)
Dietary patterns						
Mediterranean diet (MedDiet)						
SR-MA of 3 RCTs	MedDiet intervention vs control diet	Sample size ranged from 605 to 7,447 (follow-	Adults with T1D or T2D, ≥1 year follow-up, records of	Beneficial effect on:	2020	(Becerra-Tomás et al. 2020)

		up from 2 to 4.8 years)	incidend/mortality from CVD, CHDm MI and strokes.	CVD incidence RR=0.62 (95% CI: 0.5–0.78), and MI RR=0.65 (95% CI: 0.40–0.88)		
SR-MA of 38 prospective cohort studies	Highest versus lowest categories of MedDiet adherence	Sample size ranged from 274 to 193,527 partici pants (follow-up from 2 to 26 years)		Inverse association with: CVD mortality RR=0.79 (95% CI: 0.77–0.82), CHD incidence RR=0.73 (95% CI: 0.62–0.86), CHD mortality RR=0.83 (95% CI: 0.75–0.92), stroke incidence RR=0.80 (95% CI: 0.61–0.88), stroke mortality RR=0.87 (95% CI:0.80–0.96), and MI incidence RR=0.73 (95% CI 0.61–0.88)	2020	(Becerra-Tomás et al. 2020)
SR-MA of 57 controlled trials	MedDiet or Mediterranean style diet	36,983 participants (from 10 days to 7 years of intervention)	Healthy adults	Beneficial impact on: Total cholesterol MD= -5.70 mg/dL (95% CI:-9.96− -1.43), LDL-C MD= -8.24 mg/dL (95% CI: -13.50− -2.99), HDL-C MD= 1.30 mg/dL (95% CI: 0.38 − 2.21), TG MD= -12.3 mg/dL (95% CI: -15.6 − -8.99) ↓ risk of: CVD incidence RR=0.61 (95% CI: 0.42–0.80), and stroke RR=0.67 (95% CI: 0.35–0.98)	2020	(Papadaki, Nolen-Doerr, and Mantzoros 2020)
SM-MA of 30 RCTs (PREDIMED)	MedDiet intervention compared to a low-fat diet	12,461 participants	Healthy adults or with high risk of CVD	No changes in CVD mortality HR= 0.81 (95% CI: 0.50 − 1.32) Reduction in strokes HR=0.60 (95% CI: 0.45 − 0.80) Moderate reduction of total cholesterol -0.16 mmol/L (95% CI: -0.32 − 0.00)	2019	(Rees et al. 2019)
DASH						
SR-MA of 113 prospective cohort studies	DASH scores	3,277,684 individuals	General adult population (≥18 years old, >8 years of follow-up)	Inversely associated with: all-cause mortality RR=0.80 (95% CI: 0.79 – 0.82), CVD incidence or mortality RR=0.80 (95% CI: 0.78	2020	(Morze et al. 2020)

				– 0.82), incidence of T2D RR=0.81 (95% CI: 0.78 – 0.85).		
SM-MA of 7 RCTs	Effect of the DAS diet on serum levels of inflammatory markers vs usual diet	451 participants	Healthy adults or with metabolic syndrome (intervention >8 weeks)	Decrease of serum hs-CRP levels MD= -1.01 (95% CI: -1.64– -0.38)	2018	(Soltani, Chitsazi, and Salehi-Abargouei 2018)
Portfolio diet and vegetarian diet						
SR-MA of 7 trials	Effect of the Portfolio dietary pattern on cardiometabolic risk factors vs an energy-matched control diet	439 participants	Adults with hyper-lipidemia (median age 57 years old, intervention ≥3 weeks)	Reduction of LDL-C MD=-0.73 mmol/L (95% CI: -0.89 – -0.65)	2018	(Chiavaroli et al. 2018)
SR-MA of 9 RCTs	Effect of vegetarian dietary pattern for ≥3 weeks	664 participants	Patients with T2D (median age 56 years old)	↓ HbA_{1c} MD= - 0.29% (95% CI:-0.54 – -0.12), fasting glucose MD= - 0.56 mmol/L (95% CI:-0.99 – -0.13), LDL-C MD= - 0.12 mmol/L (95% CI:-0.20 – -0.04), non-HDL-C MD= - 0.13 mmol/L (95% CI:-0.26 – -0.01)	2019	(Viguiliouk et al. 2019)
SR-MA of 21 cross-sectional studies	Effect of vegan and vegetarian diets vs omnivorous diet on inflammatory markers	8,270 participants (omnivores and vegans or vegetarian at least one year)	Adults with healthy BMI (mean age of 46.2 years old)	Vegan diet: ↓ CRP levels MD= -0.54 mg/L (95% CI -0.79 – -0.29)	2020	(Menzel et al. 2020)
SR-MA of 81 prospective cohort studies	The association of fruit and/or vegetable intake with CVD incidence and mortality	4,031,896 individuals	Healthy adults (>1 year of follow-up)	Inverse association on incidence of: CVD risk RR= 0.94 (95% CI:0.90–0.97), CHD RR= 0.92 (95% CI:0.87–0.96), stroke RR=0.88 (95% CI:0.83–0.93) Inverse association on mortality of: CVD RR= 0.87 (95% CI:0.85–0.90), CHD RR= 0.86 (95% CI:0.83–0.89), stroke RR=0.94 (95% CI:0.90–0.99)	2020	(Zurbau et al. 2020)

[a] The Nurses' Health Study (NHS), NHS II, and the Health Professionals' Follow- Up Study (HPFS).

CV, cardiovascular; CVE, cardiovascular event (composite of myocardial infarction, stroke, and death from cardiovascular causes); CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; MD, mean difference; MA, meta-analysis; MUFA, monounsaturated fatty acid; MI, myocardial infarction; PUFA, polyunsaturated fatty acid; RCT, randomized controlled trial; RR, relative risk; SFA, saturated fatty acids; SRREs, summary relative risk estimates; SR, systematic review; WMD, weighted mean difference.

1.3 Choline, trimethylamine N-Oxide and cardiovascular diseases

1.3.1 Trimethylamine N-oxide, an overview

TMAO is an osmolyte (molecular weight of 75.11 g/mol) that is formed by the oxidation of TMA by the hepatic enzymes FMO's (Zeisel et al. 1983). The main enzyme responsible for this oxidation is FMO3, then FMO1, FMO2, FMO5, and FMO4 in humans (Bennett et al. 2013). In cells, TMAO accumulates in response to osmotic stress and leads to the stability of folded proteins (Cho et al. 2011). This stabilizing effect might be due to an interaction directly with the protein backbone or with the side chains of proteins (Street, Bolen, and Rose 2006). It seems that the interaction with protein surfaces excludes TMAO due to a repulsive interaction in the solvation shell (Canchi et al. 2012). Furthermore, TMAO acts as an osmolyte in order to maintain the cell volume under hydrostatic stress in marine animals and in humans (Ufnal, Zadlo, and Ostaszewski 2015). In kidney's cells when concentrated urine is produced sodium and urea are accumulated which leads a high osmotic gradient; it seems that TMAO acts as an osmotic agent to protect the cells from damage caused by urea (Wang and Bolen 1997).

1.3.1.1 Trimethylamine N-Oxide metabolism

Humans produce TMAO endogenously through the formation of TMA from enzymes in the gut microbiota. As describe above dietary choline and L-carnitine are broken down to TMA by TMA-lyases (Figure 1.4) (Koeth et al. 2013; Zeisel et al. 1983). TMAO production is reduced by antibiotics but returns to normal after treatment withdrawal and the recolonization of the bacteria in the gut (Koeth et al. 2013; Tang et al. 2013). TMA-lyase is a glycyl radical enzyme encoded by Cut gene clusters present in: *Firmicutes* (36 strains), *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Lentisphaerae* (36, 8, 12, 1, and 1 strains, respectively) (Romano et al. 2015). Later, TMA is rapidly absorbed into the circulation and then excreted unchanged or

oxidized as TMAO in urine or sweat (Zeisel et al. 1983). TMA that reaches the liver is reversible oxidized to TMAO primarily by FMO3 (Bennett et al. 2013). Typically, newly synthesized TMAO is excreted into the urine within 24 hours (de la Huerga and Popper 1951; Zhang, Mitchell, and Smith 1999). There are many examples where the diet modulates the composition of the gut microbiota, and as a consequence might impact the production of metabolites (Rinninella et al. 2019). Different dietary habits, vegetarian vs omnivorous, have shown an effect on TMAO production and reported differences in fecal composition of microbiota (Koeth et al. 2013). Omnivores have higher plasma TMAO level and are enriched proportion of *Portovellas* vs *Bacteroides* (Koeth et al. 2013). On the other hand, TMAO from diet (mainly from fish) seems to be rapidly absorbed independently of the gut microbiota and rise plasma TMAO levels within 15 min (Cho et al. 2017).

TMAO is filtered by the kidneys (Bain et al. 2006). Al-Waiz et al. demonstrated that 95% of orally consumed TMA or TMAO is excreted in the urine within 24 h; only 4% of the dose was excreted in the feces and <1% in the breath (Al-waiz et al. 1987). Studies in chicken nephron have shown that TMAO is excreted in the proximal tubules via probenecid-sensitive transporter at the luminal side. However, the transport process in human nephrons has not been elucidated (Tang 2016). Several epidemiological studies have shown a positive correlation between high plasma TMAO levels and CKD (Kim et al. 2016; Mafune et al. 2016; Missailidis et al. 2016; Posada-Ayala et al. 2014). It is currently unknown if the increase in plasma TMAO in CKD patients is due solely to impaired elimination or also increased formation (Nnane and Damani 2001).

1.3.1.2 Trimethylamine N-Oxide and cardiovascular disease

Several studies have investigated the association of TMAO and CVDs risk (summarized in Table 1.3). The first study that reported an association between TMAO and CVDs was published in 2011

(Wang et al. 2011). Through a metabolomic analysis of specific molecules were identified and related to high cardiovascular events (heart attack, stroke, or death) over three years in 75 patients who underwent for a cardiac assessment (Wang et al. 2011). In a larger cohort (*N*=1,020), plasma choline, betaine, and TMAO were significantly associated with high burden of atherosclerotic plaque (Wang et al. 2011). In a follow-up study, patients (N=4007) underwent an elective cardiac catheterization diagnosis and were followed for three years. The patients in the highest quartile of fasting plasma TMAO levels showed a substantial increase in cardiovascular events (myocardial infarction, stroke, or death) (Tang et al. 2013). However, the patients from these cohorts had impaired kidney function, which impacts the interpretation of the data (Tang et al. 2013; Wang et al. 2011). Other research groups have been reported similar findings. A cross-sectional study observed that serum TMAO (but not L-carnitine) positively associated with cardiovascular disease in a multiethnic population in Canada, even after accounting for covariates, such as meat, fish, cholesterol, and energy intake (Mente et al. 2015). Another cross-sectional study (*N*=227) observed that patients in the highest quartile of plasma TMAO levels had an increased rate of coronary events following cardiovascular surgery (Mafune et al. 2016). This study also showed a clear association between advanced-stage CKD showed high plasma TMAO levels (Mafune et al. 2016). Thirty eight percent of patients in the highest TMAO quartile had stage 3 kidney disease, no patients in the lowest TMAO quartile had kidney disease (Mafune et al. 2016). Plasma TMAO concentrations were reported higher in patients with chronic heart failure (Tang et al. 2014). In a cohort (*N*=2,235) of patients with stable coronary artery disease, elevated plasma TMAO levels were associated with greater long-term mortality risk (Senthong, Wang, et al. 2016). While high plasma choline and betaine levels were associated with incident mortality, myocardial infarction, and stroke risk, independent of other traditional risk factors, but only when the increase of plasma

TMAO levels was observed (Senthong, Wang, et al. 2016). Other studies observed a strong association between plasma TMAO levels and long-term mortality in heart failure patients, with advanced left-ventricular diastolic dysfunction, and with atherosclerosis burden in patients with atherosclerotic coronary artery disease (Heianza et al. 2020; Qi et al. 2018; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Sheng et al. 2019; Suzuki, Liam M. Heaney, et al. 2017; Suzuki, Liam M Heaney, et al. 2017; W.H. Wilson Tang et al. 2015; W. H. W. Tang et al. 2015; Wang et al. 2014).

Table 1.3 Epidemiological studies studying the relationship between TMAO and CVD

Type of study	Sample size	Population	Biomarkers	Outcomes	Dietary assessment of TMAO-precursors intake	Reference
Case control study	1,876	Stable individuals enrolled for cardia evaluation	Plasma choline, TMAO, and betaine (μM)	A significant choline-metabolites dose-dependent associations with the presence of CVD (myocardial infarction, stroke, or death 3-year follow-up)	No	(Wang et al. 2011)
Case control study	1,020	Adults without recent history of myocardial infarction or coronary artery bypass graft.	Plasma TMAO levels (μM)	Significant dose–response relationship between TMAO levels and clinical atherosclerotic plaque burden in subjects undergoing coronary angiography	No	(Wang et al. 2011)
Cohort study	2,595	Adult enrolled for an elective cardiac evaluation	Plasma L-carnitine and TMAO (μM)	Significant dose-dependent associations between carnitine concentration and risks of prevalent coronary artery disease, peripheral artery disease and overall CVD, but only among subjects with high TMAO levels	No	(Koeth et al. 2013)
Prospective observational cohort	4,007	Adult enrolled for an elective diagnostic cardiac catheterization (3 years follow-up)	Plasma choline, TMAO, and betaine	Participants who had major adverse cardiovascular events also had higher baseline levels of TMAO, as compared with those who did not have cardiovascular events (median, 5.0 μM [interquartile range, 3.0 to 8.8] vs. 3.5 μM [interquartile range, 2.4 to 5.9]; P<0.001)	No	(Tang et al. 2013)
Longitudinal study cohort	3,903 individuals	Adults undergone for an elective diagnostic coronary angiography (3 years of follow-up)	Fasting plasma choline and betaine levels and risk of major adverse	Positive correlations between plasma levels of TMAO and choline (r=0.33, P<0.001), and	No	(Wang et al. 2014)

			cardiac events in relation to TMAO	TMAO and betaine (r=0.09, P<0.001) Positive association between higher plasma choline and betaine levels (1.9- and 1.4-fold) with risk increase of major adverse cardiac events		
Longitudinal study cohort	720 individuals	Adults with stable heart failure (5 years of follow-up)	Fasting plasma TMAO levels and all-cause mortality	Significant association between higher plasma TMAO levels (3.4-fold) and high risk of mortality	No	(Tang et al. 2014)
Cross-sectional study	30 individuals 15 cases	Cases of CKD	Plasma and urine TMAO	↑ plasma TMAO levels in individuals with CKD	No	(Posada-Ayala et al. 2014)
Cohort study	475 subjects	Adults discharged after 4 months of acute coronary admission	Follow-up (~1,804 days) for records of secondary acute MI, admission for heart failure, unstable angina and all CVE	Patients with diabetes: ↑ plasma betaine associated with heart failure and all CVE ↑ TMAO associated with death, MI, heart failure, unstable angina, and all CVE Patients without diabetes: ↓ plasma betaine associated with secondary MI, unstable angina, and all CVE ↑ TMAO associated with death, and heart failure	No	(Lever et al. 2014)
Longitudinal observational case and control study cohort	521 cases 3,166 control	Adults with or without chronic kidney diseases (5 years of follow-up)	Fasting plasma TMAO and all-cause mortality	↑ predictive mortality risk at ↑ TMAO levels (HR=1.93; 95% CI=1.13–3.29; P<0.05).	No	(W. H. W. Tang et al. 2015)
Observational case and control study cohort	7 cases 6 control	Subjects on chronic hemodialysis vs normal control adults	Plasma TMAO levels	Individuals in hemodialysis had higher peak predialysis plasma TMAO levels than controls (77 ± 26 vs 2±1 μM, mean ± SD, p<0.05)	No	(Hai et al. 2015)

Cross-sectional study	1,286 individuals	In 292 consecutive individuals (99 CVD cases and 193 unmatched control subjects)	Fasting L-carnitine and TMAO levels	No association between L-carnitine levels and prevalent CVD Significant association between TMAO levels and CVD (odds ratio, 3.17; 95% CI: 1.05-9.51; P=0.02). No association between carotid intimal medial thickness and L-carnitine (P=0.64) or TMAO (P=0.18)	No	(Mente et al. 2015)
Prospective cohort study	112 individuals	Adults with chronic systolic HF with comprehensive echocardiographic evaluation (5 years follow-up)	Plasma TMAO, choline, and betaine	Plasma TMAO levels, choline, and betaine levels were correlated with each other [5.8 (3.6-12.1) mmol/L, 10.9 (8.4-14.0) mmol/L, and 43.8 (37.1-53.0) mmol/L, respectively] ↑ Plasma TMAO levels in patients with diabetes mellitus (9.4 [4.9-13.2] vs 4.8 [3.4-9.8] mmol/L; P=0.005) and with New York Heart Association functional class III or greater (7.0 [4.7-14.8] vs 4.7 [3.4-11.3] mmol/L; P=0.02).	No	(Tang et al. 2015)
Prospective cohort study	235 cases	Patients receiving HD and measured TMAO in pooled serum from healthy controls.	Serum TMAO levels, nutritional and cardiovascular risk factors Time to death to CV death or hospitalization	↑ Serum TMAO in patients under hemodialysis (median=43 mM/L; 25th-75th percentile, 28-67 mM/L) compared those with normal or near-normal kidney function (1.41±0.49 mM/L) Direct correlation between TMAO and serum albumin, prealbumin, and creatinine Inverse correlation between TMAO and log C-reactive protein	No	(Kaysen et al. 2015)

				No association between high TMAO and time to death or to CV hospitalization or CV death		
Cohort study	339 patients	Adults patients undergoing coronary angiography for the evaluation of suspected coronary artery disease.	Plasma choline, betaine, and TMAO levels	↑ TMAO in patients with diabetes compared to euglycemic patients and in patients with metabolic syndrome compared to patients without metabolic syndrome Plasma TMAO or choline increased significantly with decreasing renal function Plasma levels of choline were significantly lower in patients with a history of acute myocardial infarction as compared to those without such history	No	(Mueller et al. 2015)
Prospective cohort study	2,235 patients	Adults with stable coronary artery disease undergoing an elective coronary angiography (5 years of follow-up)	Fasting plasma TMAO and all-cause mortality	Association between ↑ plasma TMAO and mortality risk	No	(Senthong, Wang, et al. 2016)
Case-control study and a dietary intervention	283 individuals	Diabetes case-control study and a vitamin-supplementation trial (baseline parameters)	Plasma TMA, TMAO, choline, lipids, phospholipids, and methyl metabolites	Association between T2DM and high plasma TMAO concentration Inverse association between plasma HDL-C and high plasma TMAO concentration	No	(Obeid et al. 2016)
Longitudinal case-control study	85 controls 179 cases	Cases: patients with CKD (5 years of follow-up)	Plasma TMAO, choline, betaine Comorbidities, nutritional status, biomarkers of inflammation and GFR	↑ TMAO and CRP in CKD patients No changes on choline levels ↓ betaine levels in CKD patients ↓ survival rate in CKD patients with the highest vs the lowest tertile of TMAO levels	No	(Missailidis et al. 2016)

				↑ all-cause mortality with ↑TMAO levels (6.3-fold)		
Prospective observational cohort study	2,529 patients	Patients with stage 3 and 4 CKD to ischemic CV events (3 years follow-up)	Plasma TMAO Records of CV events	Independent association between TMAO and CV events (HR=1.23; 95% CI:1.06–1.42) ↑ TMAO quartile–↑risk of CV (adjusted HR 1.59; 95% CI: 1.04–2.43)	No	(Kim et al. 2016)
Case-control study (CCS)	60 cases 35 controls	60 Biopsy-proven NAFLD cases and 35 controls (18–60 years)	Serum levels of TMAO, choline and betaine	Adverse associations of TMAO, choline and betaine/choline ratio with scores of steatosis and total NAFLD activity	No	(Chen et al. 2016)
Cross-sectional study	1,628 individuals	Healthy adults (40–75 years)	Blood test and ultrasonographic NAFLD evaluation	Independent correlation between ↑ severity of NAFLD and ↑TMAO	No	(Chen et al. 2016)
Longitudinal observational cohort study	817 individuals	Adults (aged 33-55 years, 10-year follow-up)	Plasma TMAO	TMAO was not associated with 10-year CAC incidence Positive association between TMAO levels and egg consumption	No	(Meyer et al. 2016)
Cross-sectional study	227 patients	Patients undergoing CV surgery for coronary artery disease, valvular heart disease, or aortic disease	Serum TMAO levels Number of infarcted coronary arteries	↑ TMAO levels in advanced-stage CKD ↑ number of infarcted coronary arteries in the highest quartile and quintile of TMAO	No	(Mafune et al. 2016)
Prospective cohort study	353 individuals	Patients with atherosclerotic (coronary artery disease) CAD detected by elective coronary angiography	Fasting plasma TMAO and quantification of atherosclerotic burden (SYNTAX)	Positive correlation between of plasma TMAO and higher score of atherosclerotic burden	No	(Senthong, Li, et al. 2016)
Longitudinal observational study	1,079 patients	Adults with acute myocardial infarction	all-cause mortality or reinfarction for short (6- month) and long (2-year) term	Plasma TMAO levels predicted death/myocardial infarction at 2 years (HR 1.21; 95% CI: 1.03–1.43; P=0.023), not at 6 month	No	(Suzuki, Liam M Heaney, et al. 2017)

Two prospective cohorts	1st study in 78 participants 2nd study in 593 participants	Patients with first-ever ischemic stroke	Plasma TMAO levels Record of myocardial infarction, recurrent stroke, and cardiovascular death (1-year follow-up)	↑ plasma TMAO levels correlated with ↑ incidence of CVE Positive correlation between TMAO and proinflammatory intermediate (CD14++CD16+) monocytes	No	(Suzuki, Liam M Heaney, et al. 2017)
SR-MA of 11 prospective cohort studies	10,245 individuals	Patients undergoing elective coronary angiography, patients with CAD, patients with HF and patients with CKD (0.5 to 6.1 years of follow-up)	Plasma TMAO levels and overall mortality and CVE, and CV hospitalization and mortality	↑ TMAO was associated with a 23% higher risk of CVEs and 55% higher risk of all-cause mortality	No	(Qi et al. 2018)
Two cohorts	335 cases 53 controls	Patients with ST-segment elevation myocardial infarction	Plasma TMAO levels Coronary atherosclerotic burden (number of diseased coronary arteries and the SYNTAX score)	↑ plasma TMAO levels in cases than controls ↑ TMAO levels predicted both a high SYNTAX score (OR:1.16; 95% CI: 1.06-1.29; p=0.001) and multivessel disease (OR:1.15; 95% CI 1.01-1.32; p=0.035)	No	(Sheng et al. 2019)
Prospective case-control study	760 individuals 380 cases of CHD	Healthy women at baseline (changes after 10 years)	Plasma TMAO at baseline and after 10 years Record of cases Scores of dietary patterns assed and reported as Alternate Healthy Eating Index and the healthful plant-based diet index	TMAO levels were associated with ↑ CHD risk, ↑ ΔTMAO-CHD relationship with unhealthy patterns	No	(Heianza et al. 2020)
Prospective study	1,981 patients	Patients with suspected stable angina pectoris (7.5 years of follow-up)	Dietary choline obtained with FFQ Risk of incident acute myocardial infarction	Association between ↑ total choline, PC, and SM and ↑ risk of incident acute myocardial infarction	Yes Total choline: ~288 g/d	(Van Parys et al. 2020)

				Association between ↑ choline intake and ↑ plasma TMAO levels		
Sixteen cohort studies	32,166 participants	Participants without cardio- vascular disease, cancer, chronic kidney disease, or inflammatory bowel disease	Habitual food intake using FFQ Plasma TMAO concentration Cardiometabolic biomarkers	Positive association between circulating TMAO and intakes of animal protein, saturated fat, shellfish, total fish, eggs, and red meat intake. Negative association between circulating TMAO and plant and nuts intake	No	(Yang et al. 2021)

1.3.2 Dietary choline moieties and trimethylamine N-oxide supplementation: impact on CVD

1.3.2.1 Animal studies

The effect of dietary TMAO-precursors and the mechanism by which TMAO might be involved in CVD have been investigating in several animal studies (summarized in Table 1.4). The first studies used the apolipoprotein E knockout mice (*ApoE*$^{-/-}$mice) (Koeth et al. 2013; Wang et al. 2011). *ApoE*$^{-/-}$ mice fed dietary choline, L-carnitine, or TMAO supplementation had higher atherosclerotic plaque size, and plasma TMAO levels compared to their controls (Koeth et al. 2013; Wang et al. 2011). As well, it was found that diet supplemented with choline, betaine, or TMAO enhanced foam cell by increasing cell surface expression of two proatherogenic scavenger receptors: cluster of differentiation 36 (CD36) and scavenger receptor A in peritoneal macrophages from *ApoE*$^{-/-}$ mice (Wang et al. 2011). These effects were reversed with antibiotic treatments, suggesting that the gut microbiota was required in this pathological process (Koeth et al. 2013; Wang et al. 2011). Additionally, a TMAO supplemented diet downregulated the hepatic CYP7A1 activity, the rate-limiting step in the bile acid synthesis and a major route for cholesterol elimination from the body in the reverse cholesterol transport process (Koeth et al. 2013). In mice with exacerbate pressure overload-induced heart failure, diets supplemented with choline or TMAO increased plasma TMAO levels and worsened pulmonary edema, cardiac enlargement, myocardial fibrosis, and left ventricular ejection fraction (Organ et al. 2016). In another study, wild type female mice fed with dietary choline or TMAO supplementation showed an enhanced platelet aggregation in an *ex vivo* experiment (Zhu et al. 2016). In a different atherogenic mouse model (*Ldlr*$^{-/-}$), choline supplementation raised TMAO levels and expression of inflammatory genes in vascular cells which resulted in higher platelet aggregation (Seldin et al. 2016; Zhu et al.

2016). Furthermore, there was an increase in cytokines expression and adhesion molecules in the aortas (Seldin et al. 2016). Intraperitoneal administration with TMAO showed similar effects as dietary intervention in *Ldlr*$^{-/-}$ mice (Seldin et al. 2016). In these experiments, plasma TMAO levels are typically ~5 to 10 μM in the control groups but were increased to 50-100 uM upon treatment (Table 1.4). In order to reach ~100 μM plasma TMAO (the level associated to increased CVD), one group administered TMAO via intraperitoneal injection, which increased thrombus formation compared to controls.

It should be pointed out that the association between dietary choline/carnitine, plasma TMAO and atherosclerosis has not been observed by all laboratories. For example, when transgenic cholesterol ester transfer protein (CETP) overexpressing *ApoE*$^{-/-}$ mice were fed a L-carnitine-supplemented diet, plasma TMAO levels increased as expected; however, TMAO levels showed an inverse correlation with atherosclerotic lesion size (Collins et al. 2016). Similarly, *ApoE*$^{-/-}$ mice fed a choline supplementation diet had elevated plasma TMAO levels but did not show any effect on atherosclerotic lesions size or plasma cholesterol levels (Lindskog Jonsson et al. 2018). Recently, atherogenic-prone mice (*ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$) were fed with choline supplemented diet and had an increase in TMAO levels without changes in the atherosclerotic plaque area (Koay et al. 2020). Interestingly, the authors observed that the type of fiber in the diet impacted absorption of TMA and plasma TMAO levels. Mice fed a resistant starch fiber had higher plasma and feces TMA and TMAO concentrations compared to the native wheat starch (Koay et al. 2020). The studies that showed a positive correlation between plasma TMAO and CVD risks, reported much higher plasma TMAO levels (50-100 uM) compared to those that did not (1-10 uM). These findings suggest that the processes that lead to increase plasma TMAO and the association with atherosclerosis are complex and depend on the interaction between diet and gut microbiota.

Table 1.4 Dietary interventions with TMAO-precursors or TMAO in animal models

Experimental system	Animal model and dietary supplementation	Treatment	Outcome	Plasma TMAO level reported (controls)	Reference
TMAO as a cardiometabolic risk factor					
Atherosclerosis predisposed (*ApoE*$^{-/-}$)	Betaine supplemented diets	Control diet, or control with 1, 2 or 4% betaine for 0, 7, or 14 weeks	↑ dose of betaine correlated with ↓ atherosclerotic lesion area Betaine supplementation reduced aortic expression of TNF-a in a dose-dependent	NA	(Lv et al. 2009)
Atherosclerosis predisposed female and male mice (*ApoE*$^{-/-}$)	High dietary choline and TMAO supplementation	Time of weaning (4-week-old) placed on: Control diet (0.1% choline) or control with 0.5, or 1.0% choline or 0.12 TMAO for 12 weeks	Choline supplementation increased TMAO levels and atherosclerotic plaque size Females showed ↑ FMO activity and TMAO levels however plaques were observed similarly in both sexes, introducing a confounding factor.	Males/females w/0.5% choline: 13 μM/140 μM; w/1% choline: 24 μM/215 μM w/0.12% TMAO: 23 μM/70 μM (5 μM/10 μM controls)	(Wang et al. 2011)
Atherosclerosis predisposed mice (*ApoE*$^{-/-}$)	*ApoE*$^{-/-}$ mice; high dietary choline, betaine and TMAO supplementation and antibiotics	Control diet (0.1% choline) or control with: 1.0% choline or 1% betaine0.12 TMAO for 3 weeks+3 weeks of antibiotics	Mice fed with supplemented diets had enhanced levels of scavenger receptors CD36 and SR-A1. Increase in scavenger receptors was inhibited by antibiotics. Mice at 1% choline supplemented diet resulted in enhanced foam cells and inhibited by antibiotics.	50 μM (4 h after challenge w/d9(trimethyl)-choline (baseline 0 μM) TMAO conc. after another supplementation not indicated	(Wang et al. 2011)
Atherosclerosis predisposed female mice (*ApoE*$^{-/-}$)	*ApoE*$^{-/-}$ mice; high L-carnitine and antibiotics	Control water or control water with: 1.3% L-carnitine, +/− antibiotics; standard chow diet, at least for 4 weeks	↑ plasma TMA and TMAO in the L-carnitine group without antibiotics L-carnitine did not increase other plasma pro-atherogenic biomarkers 1.8-fold increase of aortic root lesion size in the L-carnitine The difference between supplemented group and control was only 3 mice	130 μM (10 μM)	(Koeth et al. 2013)

	ApoE$^{-/-}$ mice; high L-carnitine and antibiotics	Control diet of control diet with: 1.3% L-carnitine diet or 1.3% choline in diet; +/− antibiotics; standard chow, for 8 weeks	Reduction of TMA/TMAO with antibiotics TMAO ↓ reverse cholesterol transport in aortic cells	190 µM-w/carnitine 110 µM-w/choline (10 µM)	
	ApoE$^{-/-}$ mice; high TMAO	Control diet and supplemented diet with 0.12% TMAO for 4 weeks	TMAO ↓ cholesterol transport and expression of Cyp7a1	35 µM (9 µM)	
Rats	High TMAO infusion or angiotensin	Osmotic pump infusion with saline, TMAO, low-dose Angiotensin II, or both	In normotensive rats, blood pressure was not affected by TMAO infusion In hypertensive rats (angiotensinII) the hypertensive effect was maintained with TMAO infusion	TMAO: 58 µM (0.57 µM)	(Ufnal et al. 2014)
Atherosclerosis predisposed male mice (*ApoE*$^{-/-}$)	*ApoE*$^{-/-}$ mice; high choline and TMAO	Control diet (0.08% choline) and supplemented diet with 1% choline, or 0.12% TMAO for 6 or 16 weeks	Associations between ↑ TMAO levels with ↑ tubulointerstitial fibrosis, collagen deposition and phosphorylation of Smad3. Mice fed with high choline and TMAO had kidney injury marker-1 A dose-dependent relationship between plasma TMAO levels and monitored indices of renal histopathological and functional impairment	100 µM-w/choline 40 µM-w/TMAO (5 µM control)	(Tang et al. 2015)
Female mice	High choline and TMAO supplementation and antibiotics	Control diet (0.08% choline) and supplemented diet with 1% choline, or 0.12% TMAO for 6 weeks	↑ ex vivo platelet aggregation with choline and TMAO supplementation; antibiotics suppressed this effect for the choline supplemented mice only	Not reported	(Zhu et al. 2016)
Male mice	High dietary choline and TMAO supplementation in mice with TAC surgery	Control diet and supplemented diet with 1.2% choline, or 0.12% TMAO for 3 weeks prior TAC surgery and after for 12 weeks	Pulmonary edema, cardiac enlargement, and left ventricular ejection worse with TMAO- or choline-supplemented diets	After TAC: 25.2 µM w/TMAO, 26.6 µM w/choline (1.7 µM)	(Organ et al. 2016)

			↑ myocardial fibrosis with TMAO and choline supplementation		
Female *Ldlr*$^{-/-}$ mice	*Ldlr*$^{-/-}$ high choline supplementation	Control diet and water with or without 1.3% choline	Choline water ↑ inflammatory gene expression in aortas	55 μM (9 μM)	(Seldin et al. 2016)
Male mice with partially ligated carotid artery	Partially ligated carotid artery and TMAO infusion	Osmotic pump infusion with TMAO or control for 2 weeks post ligation	Mice with the TMAO infusion ↑ inflammasome formation TMAO dose was not reported	Not reported	(Boini et al. 2017)
Male *ApoE*$^{-/-}$ mice	TMAO supplementation	control diet with or without 0.3% TMAO for 8 weeks	TMAO supplementation ↑ 2-fold atherosclerotic lesion size, and plasma TG, total cholesterol, and LDL-C (25.5%, 31.2%, 28.3%, respectively) TMAO changed the bile acid profiles (especially in serum tauromuricholic acid, deoxycholic acid and cholic acid	Not reported	(Ding et al. 2018)
ApoE$^{-/-}$ male mice	TMAO supplementation	Drinking water with or without TMAO 1 mmol/L, and high-fat diet for 8 weeks	TMAO supplementation ↑ atherosclerotic plaque size, macrophage recruitment, CD36 and proinflammatory cytokine expressions	Not reported	(Geng et al. 2018)
Male mice	Choline supplementation	Normal chow (0.2% choline) or choline-supplemented diets (1.3% choline) for 3 weeks, then an antibiotic treatment for 6 weeks	Choline supplemented diet ↑ proinflammatory Ly6C^{high} monocytes levels, and was reversed with antibiotics	Not reported	(Haghikia et al. 2018)
Male mice	MI, choline or high TMAO or choline supplementation	Before MI: control diet, choline diet (1.2%) or/and DMB (choline analogue and inhibitor) diet or a TMAO diet (0.12%) starting 3 weeks; treatment for one more week after MI	↑ Cardiac fibrosis with 0.12% and 0.24% TMAO, but not with 0.06% TMAO MI model: TMAO or choline supplementation ↓ cardiac function and cardiac fibrosis, transformed fibroblasts into myofibroblasts and	Before MI: 42.4 μMw/TMAO (8.8 μM control), 91.6 μMw/choline (6.1 μM control; 27.8 μMw/choline + DMB)	(Yang et al. 2019)

			activated the TGF-βRI/Smad2 pathway DMB inhibited the TMAO-effect		
Female mice	High L-carnitine	Water supplemented with 1.3% L-carnitine or flavonoids (oolong tea) plus antibiotic treatment	L-carnitine ↑ TMAO levels and inflammation markers Flavonoids and antibiotic treatments ↓ plasma TMAO and inflammation markers (TNF-α, E-selectin, and VCAM-1)	400 μMw/L-carnitine (26 μM with no diet; 322 μM with flavonoids 13 μM with antibiotics)	(Chen et al. 2019)
Male mice	High cholesterol diet, high choline	1% cholesterol with or without 1% choline diets	Cholesterol + choline diet ↑ plasma TMAO levels and mRNA cholesterol uptake and secretion genes (Abcg5 and g8, and Ldlr)	7.7 μM (1 μM)	(Chen et al. 2019)
Male mice	High cholesterol diet, high TMAO	1% cholesterol with low (0.12%) or high (0.3%) TMAO diets	↑ mRNA cholesterol uptake and secretion genes (Abcg5 and g8, Ldlr, Srb1)	Not reported	
Gallstone-susceptible AKR/J male mice	High TMAO lithogenic diet	Lithogenic diet with non or 0.3% TMAO supplementation	↑ 70% gallstones and hepatic Abcg5 and g8 expression	23.3 μM (1 μM lithogenic diet)	
TMAO with protective or neutral effects on CVD					
Atherosclerosis predisposed female and male and mice (*ApoE*$^{-/-}$)	Chow diet in *ApoE*$^{-/-}$ and wild type	Proteomics and metabolomics analysis in vessels	No difference in plasma TMAO levels in the aortas in both models Atherosclerosis progression due to increase in oxidative stress, not to increased TMAO in *ApoE*$^{-/-}$ mice^{-}	Females 0.06 Males 0.25 (not reported)*	(Mayr et al. 2005)
Male hamsters	High-fat diet	High-fat diet (normal diet plus 100 g/kg fat for 5 weeks + 200 g/kg for 12 weeks) or controls	Correlations between onset of atherosclerosis and VLDL lipids, cholesterol, and N-acetylglycoproteins Negative association between TMAO and atherogenesis.	Not reported	(Martin et al. 2009)
Male mice	High-fat diet; TMAO supplementation	Control diet with or without 25% fat +/− 0.2% TMAO for 4 weeks	TMAO- supplemented HFD ↑ fasting insulin levels and resistance, and exacerbated impaired glucose	17.5 μM (normal chow 11.9 μM; high-fat chow 12 μM)	(Gao et al. 2014)

			tolerance and MCP-1 mRNA, ↓ IL-10 mRNA in adipose tissue, and ↓ atherosclerotic lesion		
Male mice (overexpress FMO3)	Water with choline	Water supplemented with 1.3% choline chloride for 6 weeks	↑ 75% plasma TMAO levels, and hepatic and plasma lipids in mice with FMO3 overexpression	16 μM (9 μM for control transgene)	(Shih et al. 2015)
Male mice (overexpress FMO3)	High-fat diet	Low-fat or high-fat/1% cholesterol chow for 16 weeks	With both diets ↑ plasma TG, VLDL/IDL/LDL, and unesterified cholesterol With high-fat diet ↑ glucose and insulin levels, plasma TG, TC, and phosphatidylcholine in the VLDL fractions	w/high-fat/cholesterol: 2.6 μM (2.2 μM for control transgene) (difference = trend)	(Bennett et al. 2013)
Male *ApoE*$^{-/-}$ expressing human CETP	L-carnitine supplementation	L-carnitine (87 mg/kg and 352 mg/kg) for 12 week with or without methimazole (a FMO inhibitor)	↑ plasma L-carnitine and TMAO levels and ↓ atherosclerotic lesion size with high L-carnitine diet. Plasma lipid and lipoprotein levels did not change TMAO may be protective against atherosclerosis development.	0.2 ppm = 2.7 μM (0.08 ppm = 1.07 μM)	(Collins et al. 2016)
Male Fischer 344 rats	L-carnitine supplementation	Water with or without L-carnitine 0.1, 0.2 and 0.5 % (or 1, 2 and 5 g/L) for a year	↑ plasma TMAO levels with high dose L-carnitine. No changes in plasma metabolome nor atherosclerotic development with L-carnitine supplementation	25.0 μM (2.5 μM for control)	(Weinert et al. 2017)
Male spontaneously hypertensive rats	TMAO supplementation	Drinking water with or without TMAO 333 mg/L to hypertensive and normotensive rats for 9 weeks; metabolic cage for 2 days at end of study	Water with TMAO ↑ 4 to 5-fold plasma TMAO and ↓ plasma NH2-terminal pro-B-type natriuretic peptide and vasopressin, left ventricular end-diastolic pressure, and cardiac fibrosis. TMAO may be beneficial for reduction of hypertension.	16 weeks: 37.3 μM (hypertensive control: 8.8 μM) (normal control: 6.3 μM) 56 weeks: 40.9 μM (hypertensive control: 8.1 μM) (normal control: 5.2 μM)	(Huc et al. 2018)

Male germ-free or conventional *ApoE*$^{-/-}$ mice	Western diet/germ-free supplemented with choline	Control diet or Western diet with or without 1.2% choline for 12 weeks	Control diet leads to smaller aortic lesions and lower plasma cholesterol levels compared to a Western diet choline supplementation ↑ plasma TMAO levels, but not in germ-free mice Choline supplementation ↑ plasma TMAO levels, but did not affect atherosclerotic lesions size or plasma cholesterol levels The microbiota was required for TMAO production Choline-supplemented Western diet ↓ body weight and epididymal fat weight in conventional *ApoE*$^{-/-}$ only	Western diet +choline: 8 µM Chow +choline: 21 µM (Western diet: 0.5 µM Chow: 1 µM) in conventional *ApoE*$^{-/-}$ mice No TMAO in germ-free mice	(Lindskog Jonsson et al. 2018)
Male rats with steatohepatitis	High-fat high-cholesterol diet to induce steatohepatitis	High-fat high-cholesterol for 16 weeks and TMAO (120 mg/kg/day) by oral gavage for 8 weeks	TMAO treatment ↓ hepatic and serum levels of cholesterol, ↓NPC1 and ↑ ABCG5/8 in the small intestine TMAO ↓ intestinal cholesterol absorption TMAO alters gut microbiota profile and restore the diversity of gut flora TMAO ↓ hepatic ER and cell death with cholesterol overload	Not reported	(Zhao et al. 2019)
Wild type, *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ male mice	Dietary fiber and choline supplementation	'Healthy diet' within the carbohydrate component, 35% energy form sucrose and the rest from native wheat starch or high amylose resistant starch. For 18 weeks 'Unhealthy diet' high-fat, high-cholesterol choline-	Both diets↑ plasma TMAO levels, without changes in the atherosclerotic plaque area Resistant starch fiber ↑plasma and feces TMA and TMAO concentrations compared to the native starch	In *ApoE*$^{-/-}$ mice choline: 1.6 normalized AU (0.2 normalized AU) In *Ldlr*$^{-/-}$ choline: 1.5 normalized AU (0.5 normalized AU) In wild type resistant starch: 1.5 normalized AU (native starch: 0.5 normalized AU)	(Koay et al. 2020)

	supplemented diet (3% Choline). For 6 weeks	

1.3.2.2 Human studies

The effect of dietary TMAO-precursors on CVDs biomarkers in humans has been less explored (summarized in table 1.5). However, these studies did not assess the intake of any TMAO-precursors. A recent study in Norway reported an association between dietary choline, including choline forms, and risk of incident acute myocardial infarction in patients with suspected stable angina pectoris (*N*=1,981) (Van Parys et al. 2020). The individuals in the highest quartile of total choline intake had the highest plasma TMAO levels (Van Parys et al. 2020). Nevertheless, the database used to assess choline intake (USDA) does not contemplate choline content of Norwegian food items.

Observational cohorts have shown that an average daily intake of ~300 mg of total choline does not associate with CHD events or CVA (cerebrovascular accident) (Bidulescu et al. 2007; Dalmeijer et al. 2008; Meyer and Shea 2017; Nagata et al. 2015). As previously mentioned, choline is one of the dietary TMAO-precursors that is present in eggs (mainly as PC). It has been reported that intake up to 6 eggs yolks (~714 mg of choline) leads to a high variability increase in a postprandial plasma TMAO, which peaks around 8 h, before returning to baseline values after 24 h (Miller et al. 2014). Another study reported that the consumption up-to 6 whole large eggs (~760 mg of total choline) for 4 weeks did not change plasma TMAO levels in 15 healthy lacto-ovo-vegetarian women (West et al. 2014). In 40 healthy participants, the consumption of up to 3 eggs for 4 weeks increased plasma levels of HDL-C and decreased plasma LDL-C and LDL-C/HDL-C levels. Plasma choline increased dose-dependently with egg intake; however, plasma TMAO levels did not change (DiMarco et al. 2017).

Subsequent studies used different dietary TMAO precursors, such as meat, fish, or choline supplement (choline bitartrate) to investigate the effect on plasma TMAO levels. In 50 healthy

volunteers, consumption of an egg-based breakfast compared to an oatmeal breakfast increased HDL, LDL, apolipoprotein concentrations, and plasma choline; however, plasma TMAO levels did not change (Missimer et al. 2018). Another crossover study provided meals with eggs, beef, fish, or fruit (as control meal) to 40 healthy men and it was found that among the meals, the fish significantly increased urine and plasma TMAO (peak within 15 min) and TMA (Cho et al. 2017). Two different studies compared egg consumption to a choline supplement (choline bitartrate tablet). For a period of 4 weeks breakfast with ~400 mg of total choline from eggs or choline supplement (in 30 participants) showed that the egg breakfast increased plasma total cholesterol, HDL-C, LDL-C , ApoA-I, ApoE, and choline, but did not alter plasma ApoB or TMAO (Lemos et al. 2018; Lemos et al. 2018b). Similar results were reported in another crossover study, where 23 volunteers consumed for 4 weeks 3 eggs/d or choline bitartrate supplement in their breakfast (DiBella et al. 2020; Thomas et al. 2020).

Besides eggs and meat, milk is another good source of choline. A cross-sectional study reported a positive correlation between milk and dairy consumption and plasma TMAO and TNF-α levels (Rohrmann et al. 2016). However, two crossover trials reported that meals with yogurt or cheese reduced postprandial TMAO in plasma and urine compared to acidified milk consumption (Burton et al. 2020). These results might be explained by the effect of acidified or fermented dairy products on the distribution and composition of the gut microbiota (Burton et al. 2017). The consumption of foods rich in choline has been widely investigated and their effect on TMAO clearly depends on the interaction with the gut microbiota; however, foods rich in TMAO, like fish are part of a protective-CVD diet (discussed previously in section 1.2.3). A cross-sectional study reported an association between fish consumption, and both urine and plasma TMAO levels (Krüger et al. 2017). In addition to the TMAO-precursors naturally present in the diet, different choline

supplements have shown a greater effect on TMAO metabolism. A study performed in vegetarians vs omnivores with an oral choline bitartrate supplement showed an increase in plasma TMAO and platelet aggregation response (Zhu et al. 2017). In 37 men, the postprandial effect on TMAO was analyzed in response to a meal supplemented with either PC or choline bitartrate (~600 mg of total choline), plasma and urinary TMAO was elevated with choline bitartrate compared to control and PC (Cho et al. 2020).

It has been well established that diet plays a key role in the prevention/development of CVDs. Recent studies have investigated the association between of choline intake in atherosclerosis. At the beginning of the 2010's, it was proposed that TMAO, a choline-gut microbiome dependent metabolite, might be a biomarker for CVD risks. Animal studies have shown that high choline diets (up to 10X of choline) increase plasma TMAO concentrations (in both $Apoe^{-/-}$ and wild-type mice) (Bennett et al. 2013; Organ et al. 2016; Tang et al. 2014; Tang et al. 2015). However, others have shown that low-dose TMAO treatment might provide a protective effect on heart dysfunction and atherosclerosis (Huc et al. 2018; Lindskog Jonsson et al. 2018). Animal studies have only used FC (choline bitartrate or choline chloride) in the diet. Nevertheless, in the North American population, two of the major moieties of choline are PC and FC (Lewis, Subhan, et al. 2014; Yonemori et al. 2013). Recently, dietary trials suggest that up to daily consumption of three eggs (rich in PC) might not be associated to CVD risk factors nor increase them in individuals with diabetes (DiMarco et al. 2017; Miller et al. 2014; Missimer et al. 2018; Richard, Cristall, et al. 2017). Interestingly, it has been reported that foods rich in choline, carnitine or TMAO content (such as eggs, fish) increase postprandial plasma TMAO levels (Cho et al. 2017, 2020; Hagen et al. 2020; Miller et al. 2014). However, the diets that were compared in these trials were not well balanced for energy, fat, or fibre content. Due to the complexity of the choline-moieties

metabolism, the forms and sources of choline in the response in TMAO production and its effect on atherosclerosis should be examined.

Table 1.5 Dietary interventions with TMAO-precursors or TMAO in humans

Type of study	N	Population	Outcomes	Intervention	Results	Assessment of TMAO-precursors intake	Reference
Prospective cohort study	14,430 individuals	Middle-aged men and women (14 years follow-up)	Choline and betaine intake (semi-quantitative FFQ) Incidence of CHD	Observational	No association between dietary choline nor betaine intake and incidence of CHD	Yes Choline (mg/d): 332 in men and 294 in women Betaine (mg/d): 118 in men and 102 in women	(Bidulescu et al. 2007)
Prospective cohort study	16,165 women	Adult women without prior CVD (aged 49-70 years, 97 months of follow-up))	Choline and betaine intake (validated FFQ) Records of CHD events and CVA (cerebrovascular accident)	Observational	No association between dietary choline nor betaine intake and CVD ↑ choline associated with ↓ homocysteine	Yes Choline (mg/d): 300±51 Betaine (mg/d): 241±74	(Dalmeijer et al. 2008)
Dietary intervention	40 participants	Healthy participants	Visit 1: PC challenge (2 hard-boiled eggs and 10 min after 250 mg deuterium [d9]-labeled PC, then 6 subjects received oral antibiotic for 1 week Visit 2: After antibiotic receiving PC challenge	Dietary intervention	Plasma and urine TMAO and d9-TMAO levels immediately detectable after the PC challenge (peak after 1 h) and were suppressed after 1 week of antibiotics treatment	No Datta only from PC challenge Choline from 2 eggs: ~250 mg	(Tang et al. 2013)

			Visit 3: 1 month without antibiotics last PC challenge Plasma choline, betaine, TMAO and d9-TMAO levels (0, 1, 2, 3, 4, 6, and 8 hours) TMAO from 24-hour urine collection				
Single blind, randomized, crossover-feeding study	15 participants	Healthy lacto-ovo-vegetarian women of reproductive age	8 weeks of (4 week of washout period in between): a) 6 n-3 enriched eggs/w b) 6 nonenriched eggs/w c) egg-free control phase Plasma choline metabolites measured before and after each intervention 3 randomly administered 24-h recalls from each intervention	Dietary intervention	Egg interventions vs egg-free control: ↑ Plasma choline and betaine levels, No change in plasma TMAO levels	Yes Choline and betaine (mg/d): a) 263 and 183 at n-3 enriched egg b) 265 and 255 at nonenriched egg	(West et al. 2014)
Double-blind, randomized dietary intervention	Breakfast doses of 0, 1, 2, 4, or 6 egg yolks.	6 healthy volunteers (age range: 28–53 years old)	Controlled diets on the day before and day of each egg dose with a standardized low-choline menu.	Plasma post-prandial TMAO (0, 1, 2, 4, 8, and 24 h after each intervention)	Association of consuming ≥ 2 eggs with increased plasma and urine TMAO, ~14% of the total choline in eggs having been converted to	No	(Miller et al. 2014)

				24-h urine TMAO (pre-dose and 24-h post-dose)	TMAO. Considerable variation among individuals in the TMAO response.		
Prospective cohort study	29,079 individuals	Healthy adults (aged ≥35 years)	Records of deaths from CHD and stroke (over 16 years of follow-up)	Dietary intake assessment at baseline (validated FFQ)	Betaine intake was associated with low risk of mortality from CHD in men No association between betaine intake and risk of mortality from ischemic stroke No association between choline intake and CVD mortality risk	Yes Intake (mg/d) of betaine: ~327 in men and ~268 in women, and choline: 484 in men and 416 in women	(Nagata et al. 2015)
Randomized, controlled, crossover feeding trial	19 participants	Healthy adults (18-45 years)	28 day following high and low GL diet patterns	Plasma metabolomic analysis including TMAO	↑ plasma TMAO levels after consuming the low GL diet	No	(Barton et al. 2015)
Dietary intervention (pilot study)	17 volunteers	Healthy adults (aged 18-36)	28 days of dietary supplementation with krill oil (832.5 mg EPA and DHA/d)	Plasma choline, betaine, and TMAO (before and after supplementation)	Reduction of plasma TG/HDL-C, and n-6-/n-3 ratio Increase of plasma choline, betaine and ɣ-butyrobetaine (carnitine-precurssor) No chages of plasma TMAO and Carnitine	No Krill oil contains PC (not reported)	(Berge et al. 2015)
Cross-sectional study	271 participants		Food consumption (at least two 24-h dietary recalls)	Plasma TMAO, choline, and betaine concentrations	Association between ↑ plasma TMAO and ↑ milk and dairy consumption and ↑TNF-α No association between plasma TMAO, choline or betaine and meat,	No	(Rohrmann et al. 2016)

					egg or fish consumption		
Cross-sectional study	297 individuals	Healthy adults	Daily food consumption summarized 35 food groups	24 h dietary recall Blood and urine samples collected	Association between fish consumption and urine and plasma TMAO levels Association between meat consumption and plasma TMAO levels	No	(Krüger et al. 2017)
Dietary intervention	18 participants	Healthy adults (46±5 years of age) 8 vegans/vegetarian and 10 omnivores	Prior 1 month without antibiotics or probiotics Oral choline bitartrate supplementation (500 mg twice/d) for 2 months	Plasma TMAO levels Platelet function	↑ >10-fold plasma TMAO and ↑ platelet aggregation response in both vegan/vegetarian and omnivores after intervention Positive association between plasma TMAO and platelet function	No Total choline only from supplement 450 mg/d	(Zhu et al. 2017)
Dietary intervention	122 volunteers	Elderly women (over 60 years)	Dietary supplementation with folic acid 400 µg/d for 8 weeks	Dietary pattern estimation (FFQ) Antropometrics Plasma FC, L-carnitine, TMA and TMAO	↑ plasma L-carnitine, FC, and TMA in women with western style dietary pattern Choline or betaine did not affect plasma FC, TMA, or TMAO Positive correlation between BMI and FC and TMA	No reported	(Malinowska, Szwengiel, and Chmurzynska 2017)
Crossover dietary intervention	40 participants	Healthy adults (aged 18-30 years)	2-week washout period of no egg consumption Following the consumption of 1,	Dietary records, and plasma biomarkers (lipids, glucose, choline, and TMAO)	Compared to no egg consumption: 1, 2, and 3 eggs/day ↑ HDL-C, ↓ LDL-C and ↓ LDL-C/HDL-C	Yes Choline intake (mg/d):	(DiMarco et al. 2017)

			2, and 3 eggs/d for 4 weeks each		Plasma choline increased dose-dependently with egg intake, but no TMAO	0 egg: ~324, 1 egg: ~421, 2 eggs ~563, and 4 eggs: ~696	
Randomized, controlled crossover feeding trial	40 participants	Healthy young men	Study meals: (i) eggs (3 whole hard boiled (ii) beef (6 oz), (iii) fish (cod fillet, 6 oz), and (iv) fruit control 1 week of washout period	24-h dietary recall Blood samples after meal (0, 15 and 30 min, and 1, 2, 4, and 6 h) Urine sample Stool sample	Among the meals, fish meal showed ↑ urine and plasma TMAO (peak within 15 min), plasma TMA and dimethylamine.	No Only from meals Choline content (mg): Egg: ~479, beef: ~132, fish: ~161, and fruit: ~3.8 Betaine content (mg): Egg: ~0.9, beef: ~12.7, fish: ~12.4, and fruit: ~3.8 TMAO content (mg): Egg: ~0.8, beef: ~0.9, and fish: ~529	(Cho et al. 2017)
SR-MA of 6 prospective studies	184,010 individuals	Adults (>18 years), general population	CVD outcomes (incidence and mortality)	Dietary choline and betaine	No association between incident CVD and choline (RR=1.00; 95% CI: 0.98-1.02) or betaine (RR=0.99; 95% CI: 0.98-1.01) intake No association between CVD mortality and choline intake (RR=1.09, 95% CI: 0.89-1.35)	NA	(Meyer and Shea 2017)

Crossover feeding study	40 participants	Healthy young men	Consumption of low choline and TMAO meal with d9-TMAO (50 mg) tracer	24-h dietary recall Blood samples after meal (0, 15 and 30 min, and 1, 2, 4, and 6 h) Urine sample Stool sample	Plasma d9-TMAO was detected at 15 min, reached peak at 1 h and remained elevated through the 6-h period. Estimated turnover time of TMAO of 5.3 h, ~96% of the dose eliminated in urine by 24 h. No d9-TMAO was detected in feces	No	(Taesuwan et al. 2017)
Randomized crossover clinical intervention	50 participants	Young healthy adults	Breakfast for 4 weeks: (i) 2 eggs/d (ii) 1 packet of oatmeal/d 3-week washout period in between	Fasting plasma (after each intervention) 3-day dietary intake records	Egg vs oatmeal breakfast: ↑ large HDL, large LDL, apolipoprotein concentrations. ↑ dietary and plasma choline. No change in plasma TMAO	Yes Choline (mg/d): eggs: ~450 oatmeal: ~300	(Missimer et al. 2018)
Crossover intervention	30 participants	Healthy adults (aged 18-30years)	For 4-week consuming breakfast with ~400 mg of total choline from: (i) 3 eggs/d or (ii) choline bitartrate supplement 3-week washout period	Plasma choline and TMAO (baseline and end of each intervention) 3-day dietary intake records	Eggs vs choline supplement: ↑ SFA and MUFAs intake ↑ ~7.5% total cholesterol, ~5% HDL-C, ~8.1% LDL-C, ~8% apoA-I, and ~17% apoE ↑ ~20% of plasma choline No change in plasma LDL-C/HDL-C ratio, apoB, and TMAO	Yes Choline (mg/d): eggs: ~700 choline supplement: ~690	(Lemos et al. 2018; Lemos et al. 2018b)

					Choline from egg appears to be more bioavailable		
Randomized cross-over intervention	23 participants	Adults with metabolic syndrome (aged 32-70 years)	2-week washout period without choline intake For 4-week consuming breakfast with ~400 mg of total choline from: (i) 3 eggs/d or (ii) choline bitartrate supplement 3-week washout period in between	Plasma choline and TMAO (baseline and end of each intervention) 3-day dietary intake records	↑ plasma choline with interventions, but no change between treatments No changes in plasma total, LDL-C, HDL-C, TG, glucose, TMAO compared either to baseline or between treatments	Yes Choline (mg/d): eggs: ~650 choline supplement: ~650	(DiBella et al. 2020; Thomas et al. 2020)
Randomized, controlled, double-blinded, crossover study,	37 participants	Healthy adult men	Tomato soup meals containing 600 mg of choline supplemented from: (i) PC (derived from soybean), (ii) choline bitartrate supplement, or (iii) control (no added choline)	Baseline stool sample For each day of intervention: Blood sample (0, 0.5, 1, 2, 4, 6 h) and urine sample	Choline bitartrate, from baseline ↑ plasma and urinary TMAO compared to control and PC meals Gut microbiota composition: High-TMAO producers had more abundant lineages of *Clostridium* from *Ruminococcaceae* and *Lachnospiraceae* compared to low-TMAO producers	No Only from meals Choline content (mg): PC: ~623, choline bitartrate: ~619, and control: ~42 Betaine content (mg): PC: ~7, choline bitartrate: ~7, and control: ~7	(Cho et al. 2020)

Randomized, cross-over trials	14 volunteers	Healthy young men	2-week intervention (400 g/d) with: (i) fermented yogurt or (ii) acidified milk 3-week washout period in between	For each day of intervention: Blood sample (0, 0.5, 1, 2, 4, 6 h) and urine sample	After yogurt consumption ↓ 6 h postprandial TMAO response (net iAUC) in plasma and urine compared to acidified milk consumption No changes in choline nor betaine levels	No	(Burton et al. 2020)
Randomized, cross-over trials	11 participants	Healthy young adults	Test three isocaloric test foods: (i) 600 mL milk, (ii) 100 g Gruyère cheese (plus 500 mL still water) and (iii) 600 mL soya drink (selected as a non-dairy food) After each intervention, a standardized lunch and dinner was provided	For each day of intervention: Blood sample (0, 0.5, 1, 2, 4, 6, 24 h) and urine sample	After cheese consumption ↓ cumulative TMAO urinary excretion and postprandial betaine and choline response compared to milk, while plasma TMAO levels were not changed	No	(Burton et al. 2020)
Randomized, cross-over study	20 participants	Overweight, postmenopausal women	2 whole eggs and the equivalent amount of yolk-free substitute as breakfast for 4 weeks 4-week washout in between	Fasting blood draws and stool at the beginning and end of each treatment period Plasma TMAO, choline, betaine	Plasma choline and betaine were significantly increased after whole egg vs yolk-free No change in TMAO levels Large inter-individual variability in gut microbiome	No	(Zhu et al. 2020)
Randomized crossover trial	36 volunteers	Healthy omnivorous adults	1 of 2 sequences:	Fasting blood draws at the end of	↓ Plasma TMAO levels with Plant vs Animal,	No	(Crimarco et al. 2020)

	8-week of Plant followed by 8-week of Animal, or vice versa At the end of the first 8-week phase, the second phase began without washout period	each intervention period To assess adherence: Food intake for 3 days was self-recorded biweekly and 24h recalls	but only for those that received the Plant first No associations of overall gut microbiota composition with diets, diet order, or TMAO production

1.4 Research plan

1.4.1 Rationale

Choline is an essential nutrient necessary for several biological processes like cell membrane formation via phospholipid synthesis, acetylcholine biosynthesis, lipoprotein secretion, and as a source of methyl groups when it is oxidized to betaine. Choline can be obtained through *de novo* biosynthesis and diet (Institute of Medicine 1998; Zeisel et al. 1991; Zeisel and da Costa 2009). Mammals are able to synthesize choline from the *de novo* pathway which consist three consecutive methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) in the liver (Vance 2014). Nevertheless, the amount of choline synthesized *de novo* does not cover the human needs and additional choline is needed through the diet. The adequate intake (AI) is 550 and 425 mg/d for men and pre-menopausal, non-pregnant or lactating women, respectively (Institute of Medicine 1998). Choline is present in a variety of foods as different forms; phosphatidylcholine (PC), free-choline (FC), and glycerophosphocholine being the three most common in the diet of North American population (Lewis, Subhan, et al. 2014; Yonemori et al. 2013). However, several studies have reported that the intake of choline is below the AI in different populations worldwide (Anon 2016; Elizabeth N. Pearce 2016; Gao, Wang, et al. 2016; Lewis, Subhan, et al. 2014; U.S. Department of Agriculture 2020; Wallace et al. 2018; Wallace and Fulgoni 2017). In order to meet the AI it has been recommended to increase the consumption of choline rich foods like eggs, dairy, meat, soy bean, and broccoli (high in PC) (Patterson et al. 2008b). Another important source of choline are vegetable, fruit, cereal and some legumes, which are rich in FC (Patterson et al. 2008b). However, in the last decade studies have suggested that high consumption of choline rich foods (specifically PC from animal source) may increase cardiovascular disease (CVD) risk (Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; Lever et al. 2014; Mafune et al. 2016;

Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; W.H. Wilson Tang et al. 2015; W. H. W. Tang et al. 2015; Wang et al. 2011, 2014; Yang et al. 2021; Zhu et al. 2016). In theory, excess dietary PC and free-choline reaches the large intestine and is metabolized by gut microorganisms to trimethylamine (TMA) and then is further oxidized in the liver to form trimethylamine N-oxide (TMAO) (de la Huerga and Popper 1951; Lang et al. 1998; Romano et al. 2015; Wang et al. 2011; Zeisel 1981; Zhang et al. 1999). Epidemiological and animal studies suggested that high choline intake may increase cardiovascular disease risk by elevating plasma TMAO levels (Gregory et al. 2015; Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; Lever et al. 2014; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; Tang et al. 2015; Tang et al. 2015b; Wang et al. 2011, 2014; Zhu et al. 2016). Even though others have reported a beneficial effect of TMAO treatment in atherosclerosis and blood pressure (Huc et al. 2018; Lindskog Jonsson et al. 2018). It is important to highlight that most of the animal studies have used FC (choline bitartrate or choline chloride), but not PC.

In humans, some dietary interventions have reported that up to a daily consumption of three eggs might not be associated with CVD risk factors (DiMarco et al. 2017; Miller et al. 2014; Missimer et al. 2018). Meanwhile, other intervention studies that provided meals rich in choline, carnitine, or TMAO content (such as eggs, fish) have shown an increase postprandial plasma TMAO levels (Cho et al. 2017, 2020; Miller et al. 2014) and plasma TMAO levels after 8 wks of intervention (Hagen et al. 2020). However, the diets provided in these interventions were not standardized for energy, fat (amount and type) or fibre content. Clearly, the process involving the forms and source of dietary choline with TMAO response is complex and needs further investigation. As well, it

seems the duration of the dietary interventions matter to understand if it affects the TMAO accumulation in circulation.

1.4.2 Objectives and hypotheses

The overall aim of this research is to understand the role of dietary choline supplementation on atherosclerosis development. To address this overall objective, this thesis will present 3 related but independent projects:

1. The objective of CHAPTER 2 was to investigate how dietary supplementation of choline-related metabolites (choline, betaine, and TMAO) influences atherosclerosis development in 2 atherogenic mouse models [LDL receptor knockout (*Ldlr*$^{-/-}$) and Apolipoprotein E knockout (*Apoe*$^{-/-}$) mice]. We **hypothesized** that dietary choline (10X) or TMAO supplementation, but not dietary betaine, would induce atherosclerotic plaque formation by increasing plasma TMAO concentrations in male *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice.
2. The objective of CHAPTER 3 was to investigate the effect of dietary PC supplementation on atherosclerosis development in male *Ldlr*$^{-/-}$ mice. We **hypothesized** that dietary choline supplementation (4X of choline) (either as FC or PC) would not enhance atherosclerotic plaque formation in male *Ldlr*$^{-/-}$ mice.
3. The objective of CHAPTER 4 was to examine the effect of standardized breakfasts containing different choline forms (PC versus FC) on postprandial choline and TMAO metabolism in healthy men. We **hypothesized** that the source or amount of dietary choline will have no effect on TMAO production or status following a meal but will alter choline concentration in plasma.

1.4.3 Chapter format

The objectives and hypotheses stated above were tested in three studies. These studies were organized into thesis chapters that have been submitted and/or accepted for publication as individual manuscripts.

Chapter 2 reports the effect of dietary choline, betaine or TMAO on atherosclerosis development in two atherogenic mouse models (*Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice). Objective 1 was addressed in this chapter. In summary, *Ldlr*$^{-/-}$ mice fed a choline or TMAO supplemented diet for 8 wk showed an increased in plasma TMAO levels by 1.6- and 4-fold, respectively. After 16 wk of consuming TMAO diet, they had 2-fold increase in plasma TMAO. Meanwhile, *Apoe*$^{-/-}$ mice did not increase plasma TMAO concentrations when consumed choline or TMAO supplemented diet for 12 wk. However, choline and TMAO supplementation for 28 wk significantly increased plasma TMAO concentrations by 1.8- and 1.5-fold, respectively. Interestingly, any of the dietary interventions did not enhance atherosclerotic lesion size in both mouse models.

Chapter 3 reports whether the form of dietary choline, FC or PC, influences atherosclerosis development in *Ldlr*$^{-/-}$ mice. Objective 2 was addressed in this chapter. In summary, mice upon PC supplementation had significantly lower atherosclerotic lesions while having 2-fold higher plasma TMAO levels compared with control and choline supplementation. We observed that PC supplementation reduced plasma VLDL-C and APOB48, and elevated plasma HDL-C. However, VLDL secretion was not affected by dietary treatment. Mice fed PC supplementation had lower levels of circulating proatherogenic chemokines. Our study suggests that increased dietary FC or PC intake does not induce a pro-atherogenic phenotype.

Chapter 4 examines the effect of different dietary sources of choline on postprandial choline and TMAO metabolism in healthy men. Objective 3 was addressed in this chapter. In summary, the

postprandial TMAO response was not affected in healthy men after consuming a high choline meal. However, plasma TMAO levels were increased after 0.5 and 1 h of consuming a high FC breakfast compared to high PC breakfasts. Our findings suggest that acute high FC meal consumption (> 66% AI) might increase the response of postprandial plasma TMAO concentration.

Chapter 5 discusses the findings from our research and includes directions for future research.

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Chapter 2: Dietary choline or trimethylamine N-oxide supplementation does not influence atherosclerosis development in *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ male mice

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2.1 Introduction

Choline is an essential nutrient that plays an important role in different biological functions (Zeisel et al. 1991). It is required for phospholipid synthesis, cell-membrane signaling, lipoprotein secretion, acetylcholine biosynthesis, and one-carbon metabolism (Zeisel and da Costa 2009). Currently, the DRI suggests an adequate intake of 550 mg/d for men and 425 mg/d for women (Institute of Medicine 1998). Excess of dietary choline can be metabolized to betaine or methylamines [mainly trimethylamine (TMA)] by the gut microbiota, across the small and large intestine and cecum (de la Huerga and Popper 1951; Romano et al. 2015; Zeisel 1981). Likewise, dietary choline-containing compounds such as betaine are metabolized to TMA (Wang et al. 2014). TMA is rapidly absorbed by the enterocytes and is then oxidized to trimethylamine N-oxide (TMAO) in the liver by a group of enzymes called flavin-containing monooxygenases (FMOs) (Lang et al. 1998; Wang et al. 2011; Zhang et al. 1999). Circulating TMAO is cleared by the kidneys and excreted in urine (Ufnal et al. 2015).

Many studies have reported a positive correlation between plasma TMAO concentrations and cardiovascular disease (CVD) risk in humans (Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; Lever et al. 2014; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; Tang et al. 2015; Tang et al. 2015b; Wang et al. 2011, 2014; Zhu et al. 2016). Wang et al. (Wang et al. 2011) demonstrated for the first time an association between gut microbiota–dependent metabolism of dietary choline and atherosclerosis in apoE knockout (*Apoe*$^{-/-}$) mice. Furthermore, this study reported a positive correlation between plasma TMAO concentrations and atherosclerotic plaque size (Wang et al. 2011). However, the mechanisms by which TMAO may promote atherosclerosis are still unclear. It has been reported that high plasma TMAO concentrations promote foam cell formation and

platelet aggregation and reduce reverse cholesterol transport (Koeth et al. 2013; Seldin et al. 2016; Tang et al. 2013; Zhu et al. 2016). Subsequent studies have shown that dietary choline supplementation increases plasma TMAO concentrations in both *Apoe*$^{-/-}$ and wild-type mice (Bennett et al. 2013; Organ et al. 2016; Tang et al. 2014; Tang et al. 2015). Although high plasma TMAO concentrations are positively correlated to CVD, it remains unclear whether plasma TMAO concentrations are sensitive to changes in dietary choline intake in humans. Several dietary trials suggest that high intake of choline-content food may not be associated to CVD risk factors (DiMarco et al. 2017; Miller et al. 2014; Missimer et al. 2018). Therefore, the effect of betaine has been less explored. Betaine treatment in vitro and in vivo has been shown to have an anti-inflammatory and antiatherogenic effect (Go et al. 2005; Lee et al. 2013).

The aim of this study was to investigate how dietary supplementation of choline-related metabolites (choline, betaine, and TMAO) influences atherosclerosis development in 2 atherogenic mouse models [LDL receptor knockout (*Ldlr*$^{-/-}$) and *Apoe*$^{-/-}$ mice]. We hypothesized that dietary choline or TMAO supplementation would induce atherosclerotic plaque formation by increasing plasma TMAO concentrations in male *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice. Contrary to what was predicted, dietary supplementation with choline, betaine, or TMAO did not increase atherosclerosis in either model, despite increased TMAO concentrations in plasma.

2.2 Methods

2.2.1 Animal handling

All studies had the approval from the Institutional Animal Care Committee at the University of Alberta, according with guidelines of the Canadian Council on Animal Care. Male C57Bl/6J *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ (Jackson Laboratories, B6.129P2-Apoetm1Unc/J) mice were fed an unpurified standard diet (PicoLab® Laboratory Rodent Diet 5L0D∗). At 8−10 wk, mice had free access to

their designated diet for 8, 12, 16, or 28 wk. Mice were deprived of food for 12 h and killed by cardiac puncture. Blood was collected in EDTA-containing tubes and plasma was separated by centrifugation at 3000 g for 10 min and EDTA, removed, incubated in Krebs-Henseleit buffer for 30 min, fixed stored at −80◦C. Hearts were perfused with PBS containing 5 mM overnight in 10% phosphate-buffered formalin, and cut cross-sectioned to the aortic root.

2.2.2 Diets and Feeding Trials.

Ldlr$^{-/-}$ male mice, aged 8-10 wk, were fed with high-fat diet (HFD: 40% of calories and 0.5% wt/wt of cholesterol) (*Table 2.1*) for 8 or 16 wk. Owing to the phenotype of these mice, they had to be fed with HFD with cholesterol in order to induce atherosclerosis (Getz and Reardon 2012). In the feeding trial 1 and 3, *Ldlr*$^{-/-}$ mice (*n*=5-12/group) were randomized to 1 of 3 dietary groups (*Table 2.1*): control (LC) (0.1% choline wt/wt), choline-supplemented (LCS) (1.0% choline wt/wt), or betaine-supplemented (LBS) (0.1% choline and 0.9% betaine wt/wt) for 8 (feeding trial 1) or 16 (feeding trial 3) wk. To measure the effect of high plasma TMAO levels on atherosclerosis, a couple of dietary TMAO supplementation trials were design. In the feeding trial 2 and 4, mice (*n*=7-8/group) were randomly assigned to 1 of 2 dietary groups (*Table 2.1*): LC (0.1% choline wt/wt) or TMAO-supplemented (LTS) (0.1% choline and 0.2% TMAO wt/wt) for 8 (feeding trial 2) or 16 (feeding trial 4) wk. Similarly, *Apoe*$^{-/-}$ male mice, aged 8 wk (*n*=7-8/group), were fed Teklad Global 18% Protein Rodent Diet (*Table 2.2*) for 12 (feeding trial 5) or 28 (feeding trial 6) wk (*Table 2.3*). *Apoe*$^{-/-}$ mice spontaneously develop atherosclerosis with normal low-fat rodent unpurified diet (Getz and Reardon 2012). Mice were randomly assigned to 1 of 4 dietary groups: control (EC) (0.1% choline wt/wt), choline-supplemented (ECS) (1% choline wt/wt), betaine-supplemented (EBS) (0.1% choline and 0.9% betaine wt/wt) or TMAO-supplemented (ETS) (0.1% choline and 0.12% TMAO wt/wt) (*Table 2.3*). Choline bitartrate (C1629), betaine BioUltra,

≥99.0% (NT) (CAS 107-43-7) and TMAO 95% (CAS 1184-78-7) were purchased from Sigma-Aldrich.

Table 2.1 Composition of diets in Ldlr$^{-/-}$ mice [1]

Diet ingredients	Units	LC	LCS	LBS	LTS
Protein	g (%, kcal)	272 (23)	272 (23)	272 (23)	272 (23)
Carbohydrates	g (%, kcal)	446 (38)	446 (38)	446 (38)	446 (38)
Fat	g (%, kcal)	205 (39)	205 (39)	205 (39)	205 (39)
Casein[2]	g	270	270	270	270
Corn starch[2]	g	170.65	170.65	170.65	170.65
Sucrose[3]	g	195.35	195.35	195.35	195.35
Vitamin mix (AIN-93) [2,6]:	g	19	19	19	19
Bernhart–Tomerelli mineral mix[2]:	g	50	50	50	50
Calcium phosphate dibasic[4]	g	3.4	3.4	3.4	3.4
Inositol[4]	g	6.3	6.3	6.3	6.3
Cellulose[2]	g	80	80	80	80
L-cysteine[4]	g	1.8	1.8	1.8	1.8
Cholesterol[4]	g	5	5	5	5
Lard[3]	g	155	155	155	155
Mazola corn oil[3]	g	10	10	10	10
Crisco Vegetable oil[3]	g	32	32	32	32
DHAsco[5]	g	1.5	1.5	1.5	1.5
ARAsco[5]	g	1.5	1.5	1.5	1.5
Choline bitartrate[4]	g	2.5	25	2.5	2.5
Betaine[4]	g	0	0	8.89	0
TMAO[4]	g	0	0	0	2.04

[1]Choline, betaine, and TMAO supplements were mixed to obtain 1 kg of the high-fat diet. Diet was pelleted. Feeding Trials 1, 2, 3, and 4.
[2]Ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA).
[3]Ingredients were purchased from Safeway (Edmonton, AB, Canada).
[4]Ingredients from (Sigma-Aldrich), choline bitartrate (C1629), betaine BioUltra, ≥99.0% (NT) (CAS 107-43-7) and TMAO 95% (CAS 1184-78-7).
[5] Oils were donated by DSM (Nutritional Products, Columbia, MD, USA).
[5]AIN-93-VX vitamin mix (Reeves 1997).
[6]Bernhart-Tomarelli mineral mixture (Bernhart and Tomarelli 1966).
Ldlr$^{-/-}$ mice betaine-supplemented diet (LBS), *Ldlr*$^{-/-}$ mice control diet (LC), *Ldlr*$^{-/-}$ mice choline-supplemented diet (LCS), and *Ldlr*$^{-/-}$ mice TMAO-supplemented diet (LTS).

Table 2.2 Composition of diets in Apoe$^{-/-}$ mice [1]

Diet ingredients	Units	EC	ECS	EBS	ETS
Protein	g (%, kcal)	267 (24)	267 (24)	267 (24)	267 (24)
Carbohydrates	g (%, kcal)	644 (58)	644 (58)	644 (58)	644 (58)
Fat	g (%, kcal)	89 (18)	89 (18)	89 (18)	89 (18)
Teklad Global 18% Protein Rodent Diet[2]	g	1000	979	991	999
Choline bitartrate[3]	g	0	21.41	0	0
Betaine[3]	g	0	0	8.89	0
TMAO[3]	g	0	0	0	1.22

[1]Choline, betaine, and TMAO supplements were mixed to obtain 1 kg of diet. Diets were pelleted. Feeding Trials 5 and 6.
[2]Diet no. 2018S from Harlan Teklad (Indianapolis, IN, USA). Diet composition is listed in Table 2.3.
[3]Ingredients from (Sigma-Aldrich), choline bitartrate (C1629), betaine BioUltra, ≥99.0% (NT) (CAS 107-43-7) and TMAO 95% (CAS 1184-78-7).
Apoe$^{-/-}$ mice betaine-supplemented diet (EBS), *Apoe*$^{-/-}$ mice control diet (EC), *Apoe*$^{-/-}$ mice choline-supplemented diet (ECS), and *Apoe*$^{-/-}$ mice TMAO-supplemented diet (ETS).

Table 2.3 Composition of diet Teklad Global 18% Protein Rodent Diet used in Apoe$^{-/-}$ mice diets[1]

Diet ingredients	**Units**	**EC**	**ECS**	**EBS**	**ETS**
Crude Protein	%	18.6	18.6	18.6	18.6
Fat	%	6.2	6.2	6.2	6.2
Carbohydrate	%	44.2	44.2	44.2	44.2
Calcium	%	1	1	1	1
Phosphorus	%	0.7	0.7	0.7	0.7
Sodium	%	0.2	0.2	0.2	0.2
Potassium	%	0.6	0.6	0.6	0.6
Chloride	%	0.4	0.4	0.4	0.4
Magnesium	%	0.2	0.2	0.2	0.2
Zinc	mg/kg	70	70	70	70
Manganese	mg/kg	100	100	100	100
Copper	mg/kg	15	15	15	15
Iodine	mg/kg	6	6	6	6
Iron	mg/kg	200	200	200	200
Selenium	mg/kg	0.23	0.23	0.23	0.23
Vitamin A (1 UI=0.3 µg of retinol)	IU/g	15	15	15	15
Vitamin D3 (1 UI=25 ng of cholecalciferol)	IU/g	1.5	1.5	1.5	1.5
Vitamin E	IU/kg	110	110	110	110
Vitamin K3 (menadione)	mg/kg	50	50	50	50
Vitamin B1 (thiamin)	mg/kg	17	17	17	17
Vitamin B2 (riboflavin)	mg/kg	15	15	15	15
Niacin (nicotinic acid)	mg/kg	70	70	70	70
Vitamin B6 (pyridoxine)	mg/kg	18	18	18	18
Pantothenic Acid	mg/kg	33	33	33	33
Vitamin B12 (cyanocobalamin)	mg/kg	0.08	0.08	0.08	0.08
Biotin	mg/kg	0.4	0.4	0.4	0.4
Folate	mg/kg	4	4	4	4
Choline	g/kg	1.2	1.2	1.2	1.2
Total Saturated Fatty acids	%	0.9	0.9	0.9	0.9
Total Monounsaturated Fatty acids	%	1.3	1.3	1.3	1.3
Total Polyunsaturated Fatty acids	%	3.4	3.4	3.4	3.4

[1] Diet composition was provided by Harlan Teklad lab.
Apoe$^{-/-}$ mice betaine-supplemented diet (EBS), *Apoe*$^{-/-}$ mice control diet (EC), *Apoe*$^{-/-}$ mice choline-supplemented diet (ECS), and *Apoe*$^{-/-}$ mice TMAO-supplemented diet (ETS).

2.2.3 Analysis of atherosclerosis

The top portion of the heart, with the aortic root, was incubated overnight in 30% sucrose solution and embedded in optimal cutting temperature compound (OCT; Cryomatrix Thermo Scientific) and frozen at -20°C. The aorta root was cryosectioned (10 μm thick) and stained with Oil Red O and Mayer's Hematoxylin (Sigma-Aldrich) (Zhao et al. 2009). Atherosclerotic plaque area was quantified using ImageJ from the NIH and was calculated as Oil Red O-stained area as percentage of total cross aortic surface area.

2.2.4 Lipid profile

Plasma total cholesterol and triacylglycerol (TG) concentrations were determined by colorimetric assays purchased from Wako (Catalog No. 999-02601) and SEKISUI Diagnostics (Catalog No. 236-60), respectively.

2.2.5 Choline metabolites

Plasma choline, betaine, TMA, and TMAO concentrations were measured by hydrophilic interaction chromatography for LC–tandem MS (LC-MS/MS), as previously reported (Mi et al. 2017; Xiong et al. 2012; Zhao, Xiong, and Curtis 2011). Plasma proteins were precipitated by methanol with 0.1% formic acid. The super- natant was collected and reacted with ethyl bromoacetate in the presence of ammonium hydroxide to convert TMA into ethyl betaine; subsequently LC-MS/MS analysis was used to quantify TMAO and ethyl betaine concentrations. An Agilent 1200 series HPLC system coupled to a 3200 QTRAP mass spectrometer (AB SCIEX) under turbospray positive mode was used to analyze standard and sample solutions.

2.2.6 Statistical analysis

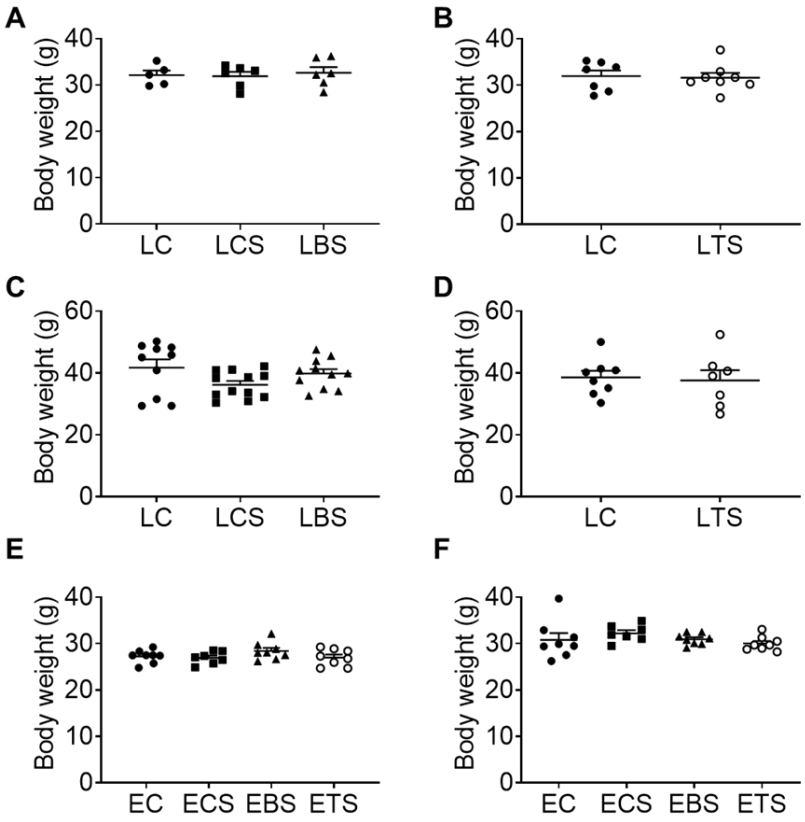
For statistical analysis Graph Pad Prism 7 was used to compare the effect of the different diets. All data were reported as mean ± SEM. For all data normal distribution was tested by Shapiro-Wilk normality test. Data that were not normally distributed were log transformed. Statistical tests were performed on the transformed data and reported as median and 95% CI. Dietary interventions were analyzed using 1-factor ANOVA followed by a Tukey's post hoc test (feeding trials 1, 3, 5, and 6) or Student's t test (feeding trials 2 and 4), when appropriate. Simple linear regression and Pearson's correlation were calculated. Significance was set at $P < 0.05$ in all analyses.

2.3 Results

2.3.1 Feeding trial 1: dietary supplementation with choline, or betaine for 8 wk did not exacerbate atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice

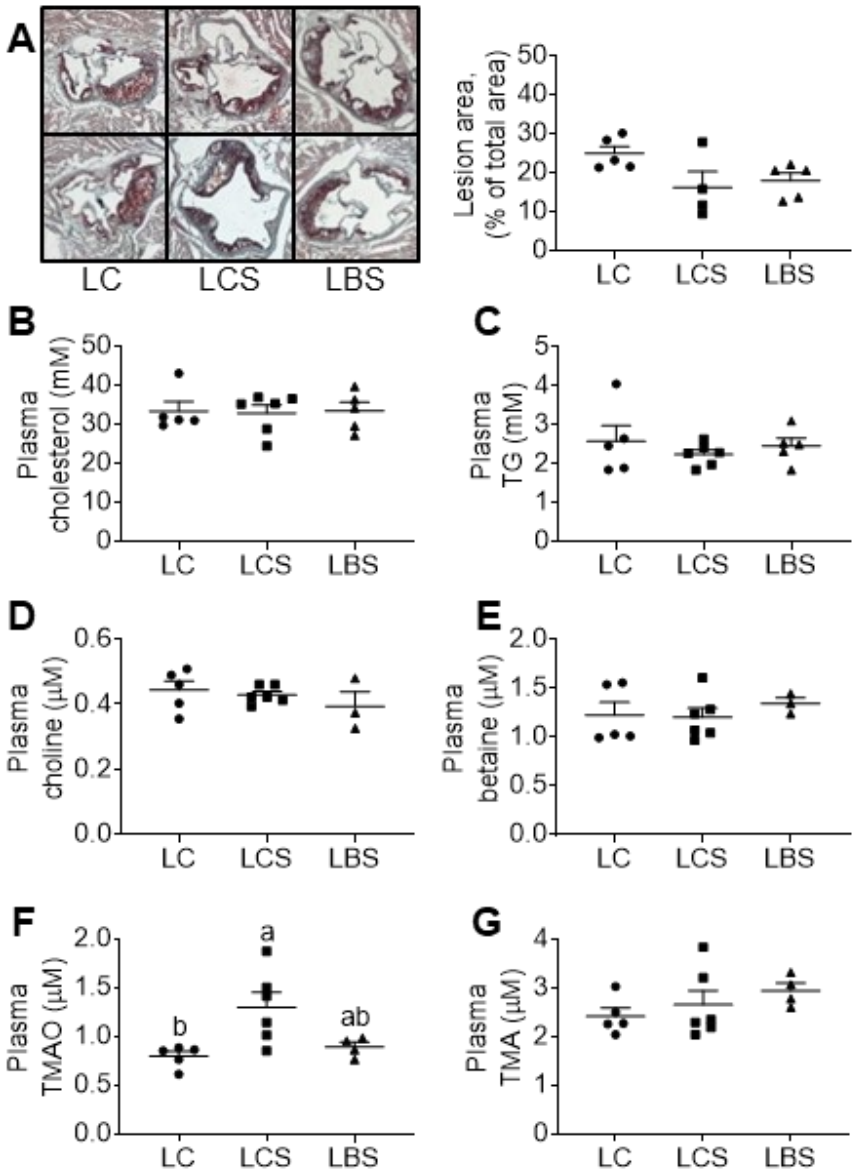
Body weight (*Figure 2.1 A*), fasting plasma choles- terol, TG, choline, and betaine concentrations were not altered by dietary choline or betaine supplementation in *Ldlr*$^{-/-}$ mice (*Figure 2.2 C-E, Table 2.4*). Surprisingly, atherosclerotic lesion size was not affected by dietary treatment (*Figure 2.2 A, B, Table 2.4*). Interestingly, plasma TMAO concentrations were 1.6-fold higher in the LCS group than in the LC group (LCS compared with LC: 1.3 compared with 0.792 μM) (*Figure 2.2 F, Table 2.4*). The concentrations of TMA were not different between dietary groups (*Figure 2.2 G, Table 2.4*). Plasma TMAO concentrations were not correlated with atherosclerotic lesion size (*Figure 2.3 A, Table 2.4*).

Figure 2.1 Body weight in all feeding trials



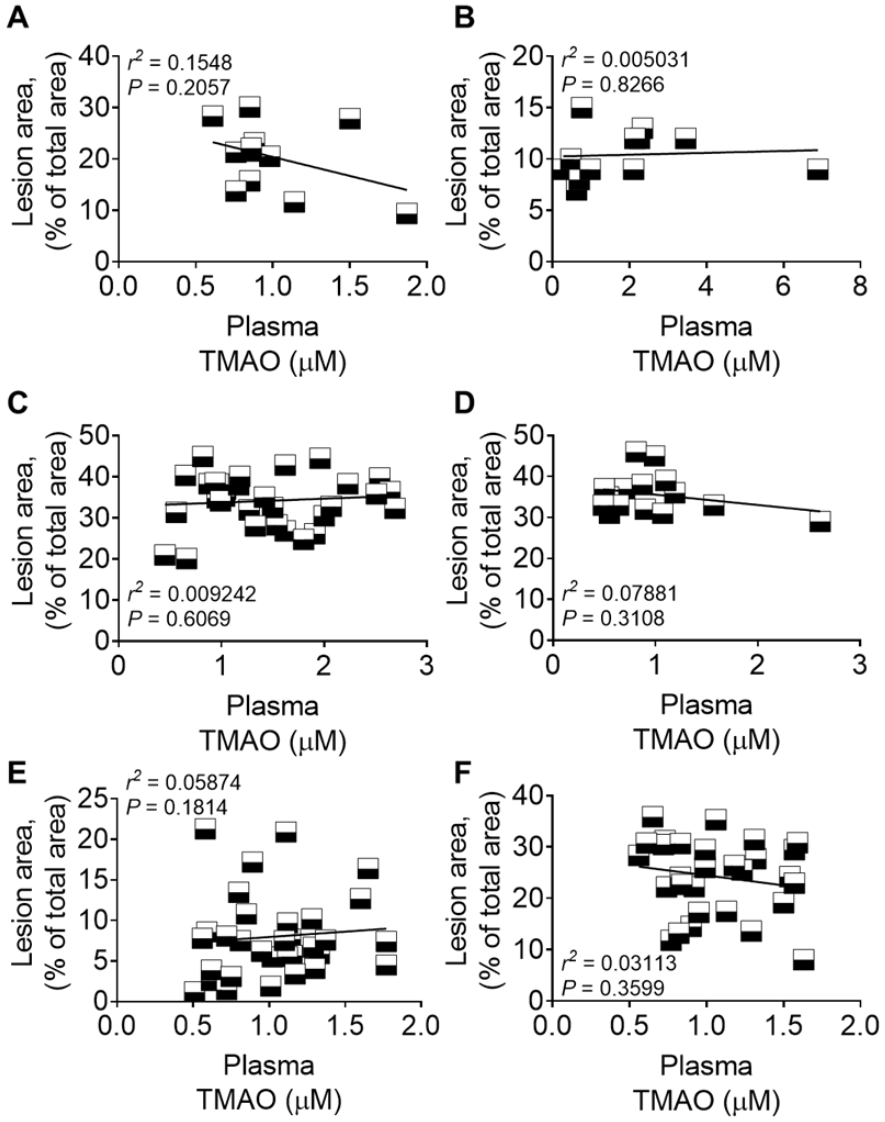
Body weight in *Ldlr*$^{-/-}$ with LC, LCS, or LBS diet for 8 weeks (Feeding Trial 1) (A), in *Ldlr*$^{-/-}$ with LC, or LTS diet for 8 weeks (Feeding Trial 2) (B), *Ldlr*$^{-/-}$ with LC, LCS, or LBS diet for 16 weeks (Feeding Trial 3) (C), in *Ldlr*$^{-/-}$ with LC, or LTS diet for 16 weeks (Feeding Trial 4) (D), in *Apoe*$^{-/-}$ mice with EC, ECS, EBS or ETS diet for 12 weeks (Feeding Trial 5) (E), and in *Apoe*$^{-/-}$ mice with EC, ECS, EBS or ETS diet for 28 weeks (Feeding Trial 6) (F). Values are means ± SEMs, n = 6-12. Groups without a common letter differ or *different from control group, $P < 0.05$. *Apoe*$^{-/-}$, Apolipoprotein E knockout; EBS, betaine-supplemented; EC, control; ECS, choline-supplemented; ETS, TMAO-supplemented; LBS, betaine-supplemented; LC, control, LCS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide. TMAO, trimethylamine N-oxide.

Figure 2.2 Dietary choline and betaine supplementation for 16 weeks in Ldlr$^{-/-}$ mice did not alter atherosclerotic size lesion



Aorta atherosclerotic lesions (A), plasma cholesterol (B), TG (C), choline (D), betaine (E), TMAO (F) and TMA (G) in 16-wk-old male *Ldlr*$^{-/-}$ mice fed with LC, LCS or LBS diet for 8wk (Feeding Trial 1). Values are means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 6. Groups without a common letter differ, $P < 0.05$. LBS, betaine-supplemented; LC, control, LCS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

Figure 2.3 Pearson correlation of plasma TMAO levels with atherosclerotic lesion size



Pearson correlation of plasma TMAO levels with atherosclerotic lesion size in *Ldlr*$^{-/-}$ with LC, LCS, or LBS diet for 8 weeks, n = 12 (Feeding Trial 1) (A), in *Ldlr*$^{-/-}$ with LC, or LTS diet for 8 weeks, n = 13 (Feeding Trial 2) (B), *Ldlr*$^{-/-}$ with LC, LCS, or LBS diet for 16 weeks, n =32 (Feeding Trial 3) (C), in *Ldlr*$^{-/-}$ with LC, or LTS diet for 16 weeks, n= 15 (Feeding Trial 4) (D), in *Apoe*$^{-/-}$ mice with EC, ECS, EBS or ETS diet for 12 weeks, n = 32 (Feeding Trial 5) (E), and in *Apoe*$^{-/-}$ mice with EC, ECS, EBS or ETS diet for 28 weeks, n= 29 (Feeding Trial 6) (F). *Apoe*$^{-/-}$, Apolipoprotein E knockout; EBS, betaine-supplemented; EC, control; ECS, choline-supplemented; ETS, TMAO-supplemented; LBS, betaine-supplemented; LC, control, LCS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

Table 2.4 Results compilation [1]

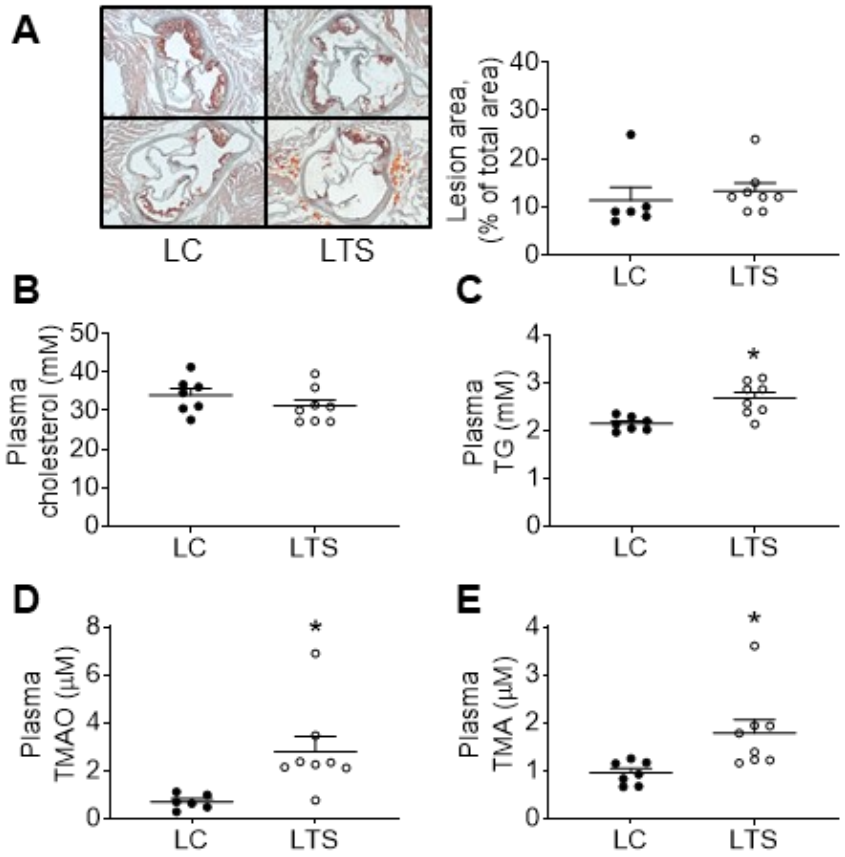
	Feeding trial 1			Feeding trial 2		Feeding trial 3			Feeding trial 4		Feeding trial 5				Feeding trial 6			
	LC	LCS	LBS	LC	LTS	LC	LCS	LBS	LC	LTS	EC	ECS	EBS	ETS	EC	ECS	EBS	ETS
Lesion area, (% of total area)	24.90 ± 1.82[2]	16.17 ± 4.11[2]	17.93 ± 1.95[2]	9 [7-25][3]	12 [9-24][3]	30.74 ± 2.46[2]	34.33 ± 1.02[2]	36.30 ± 1.95[2]	35.38 ± 1.58[2]	36.00 ± 2.16[2]	5.70 [1.20-10.20][3]	7.05 [3.82-12.83][3]	7.52 [4.44-13.87][3]	9.44 [4.03-14.26][3]	22.14 ± 3.49[2]	23.19 ± 3.00[2]	28.07 ± 1.98[2]	23.39 ± 2.02[2]
Plasma cholesterol (mM)	31.07 [29.66-43.00][3]	35.19 [24.34-36.79][3]	34.16 [27.07-39.59][3]	34.07 ± 1.74[2]	31.25 ± 1.60[2]	49.08 [20.43-54.23][3]	43.26 [20.53-53.78][3]	40.57 [29.03-51.49][3]	41.16 ± 4.65[2]	39.82 ± 4.77[2]	11.18 [7.85-13.61][3]	14.15 [8.59-15.00][3]	17.09 [14.55-19.67][3]	16.28 [10.82-19.67][3]	18.13 ± 0.93[2]	18.59 ± 1.06[2]	21.01 ± 1.08[2]	17.10 ± 1.64[2]
Plasma TG (mM)	2.57 ± 0.40[2]	2.23 ± 0.12[2]	2.45 ± 0.20[2]	2.15 ± 0.05[2]	2.68 ± 0.12[2, *]	3.80 [2.69-5.34][3]	4.21 [1.52-5.24][3]	3.75 [2.36-5.77][3]	3.66 ± 0.40[2]	4.69 ± 0.59[2]	1.06 ± 0.15[2]	0.89 ± 0.15[2]	1.16 ± 0.18[2]	1.17 ± 0.27[2]	2.96 [1.86-3.69][3]	3.22 [2.73-7.18][3]	3.82 [2.44-4.65][3]	2.94 [1.53-5.22][3]
Plasma choline (µM)	0.44 ± 0.03[2]	0.43 ± 0.01[2]	0.39 ± 0.05[2]	-	-	13.80 ± 0.41[2, b]	15.58 ± 0.60[2, b]	20.13 ± 1.39[2, a]	-	-	16.19 ± 0.63[2]	17.00 ± 0.51[2]	16.98 ± 1.24[2]	15.31 ± 0.63[2]	10.80 [9.22-14.40][3]	10.37 [9.50-13.92][3]	10.13 [8.83-13.73][3]	9.36 [9.02-10.66][3]
Plasma betaine (µM)	1.02 [0.99-1.55][3]	1.15 [0.97-1.60][3]	1.34 [1.24-1.44][3]	-	-	27.10 ± 3.45[2]	27.66 ± 3.33[2]	31.07 ± 4.29[2]	-	-	42.47 [39.52-81.01][3]	53.31 [23.73-73.33][3]	37.77 [18.69-63.77][3]	47.50 [36.79-74.44][3]	15.45 [10.24-23.05][3]	9.82 [9.13-22.37][3]	10.59 [8.88-18.78][3]	11.35 [9.22-15.54][3]
Plasma TMAO (µM)	0.79 ± 0.05[2, b]	1.30 ± 0.15[2, a]	0.89 ± 0.05[2, ab]	0.67 [0.28-1.11][3]	2.30 [0.76-6.89][3, *]	1.23 ± 0.22[2]	1.76 ± 0.20[2]	1.17 ± 0.13[2]	0.60 [0.49-0.99][3]	1.10 [0.81-2.61][3, *]	1.02 [0.51-1.33][3, ab]	1.30 [1.11-1.77][3, a]	0.80 [0.56-1.12][3, b]	0.87 [0.72-1.65][3, ab]	0.80 [0.44-1.06][3, b]	1.56 [1.13-1.63][3, a]	0.78 [0.61-1.50][3, b]	1.26 [0.84-1.57][3, a]
Plasma TMA (µM)	2.42 ± 0.17[2]	2.65 ± 0.29[2]	2.94 ± 0.16[2]	0.92 [0.66-1.25][3]	1.59 [1.16-3.60][3, *]	1.95 ± 0.27[2]	1.82 ± 0.21[2]	2.26 ± 0.24[2]	3.33 ± 0.68[2]	3.44 ± 0.68[2]	1.88 [1.06-2.47][3]	1.58 [1.07-2.24][3]	1.53 [1.10-3.50][3]	1.68 [1.29-3.19][3]	1.00 [0.41-1.59][3]	1.14 [0.88-1.66][3]	0.86 [0.68-1.88][3]	1.11 [0.74-1.75][3]

[1] Values are means ± SEMs. Groups without a common letter differ or *different from control group, $P < 0.05$.
[2] Values are means ± SEMs.
[3] Values are median [95% CI].
Apoe$^{-/-}$ mice betaine-supplemented diet (EBS), *Apoe*$^{-/-}$ mice control diet (EC), *Apoe*$^{-/-}$ mice choline-supplemented diet (ECS), *Apoe*$^{-/-}$ mice TMAO-supplemented diet (ETS), *Ldlr*$^{-/-}$ mice betaine-supplemented diet (LBS), *Ldlr*$^{-/-}$ mice control diet (LC), *Ldlr*$^{-/-}$ mice choline-supplemented diet (LCS), and *Ldlr*$^{-/-}$ mice TMAO-supplemented diet (LTS).

2.3.2 Feeding trial 2: dietary supplementation with TMAO for 8 wk did not exacerbate atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice

It is possible that these unexpected results were due to low TMA production from the microbiota; however, feeding mice an LTS diet did not affect atherosclerotic lesion size (*Figure 2.4 A, Table 2.4*), despite the fact that plasma TMAO and TMA concentrations were greater by 4- fold (LTS compared with LC: 2.8 compared with 0.7 μM) and 1.9-fold (LTS compared with LC: 0.95 compared with 1.781 μM), respectively, than in the control group (*Figure 2.4 D, E, Table 2.4*). Fasting plasma TG, but not cholesterol, concentration was increased in the LTS group (*Figure 2.4 B, C, Table 2.4*). Again, plasma TMAO concentrations did not correlate with atherosclerotic lesion size (*Figure 2.3 B*). Body weight did not differ between dietary groups (*Figure 2.1 B*).

Figure 2.4 Dietary TMAO supplementation for 16 weeks in Ldlr$^{-/-}$ mice did not alter atherosclerotic size lesion

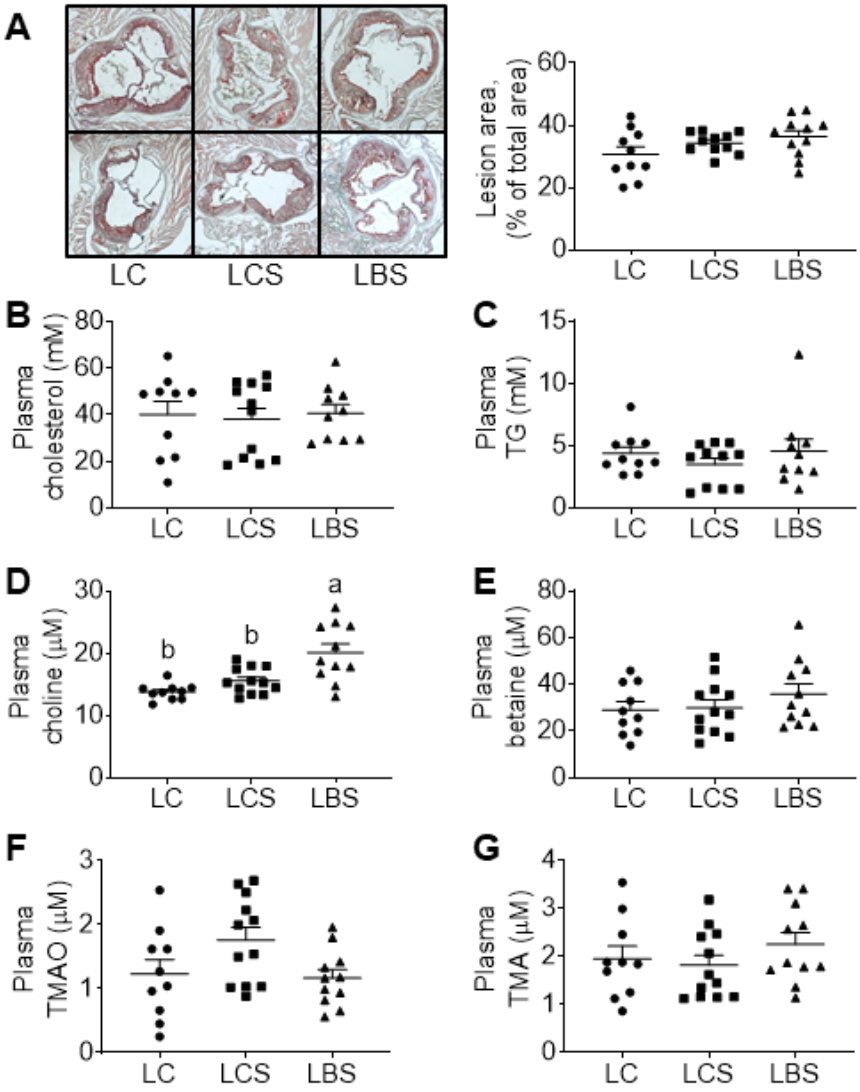


Aorta atherosclerotic lesions (A), plasma cholesterol (B), TG (C), TMAO (D), TMA (E) in 16-wk-old male *Ldlr*$^{-/-}$ mice fed with LC or LTS diet for 8wk (Feeding Trial 2). Values are means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 8. *Different from control group, $P < 0.05$. LBS, betaine-supplemented; LC, control; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; LTS, TMAO-supplemented; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

2.3.3 Feeding trial 3: dietary supplementation with choline, or betaine for 16 wk did not alter atherosclerotic size lesion in *Ldlr*$^{-/-}$ mice

Consistent with our initial experiments, dietary supplementation with choline or betaine did not differentially affect the size of atherosclerotic lesions in *Ldlr*$^{-/-}$ mice fed for 16 wk (*Figure 2.5 A, Table 2.4*). Furthermore, dietary choline or betaine supplementation did not have any effect on body weight (*Figure 2.1 C*) or fasting plasma cholesterol and TG concentrations (*Figure 2.5 B, C, Table 2.4*). Plasma choline concentrations were higher in the betaine- supplemented group than in all other groups (*Figure 2.5 D, Table 2.4*). Plasma TMA concentrations did not change between dietary groups (*Figure 2.5 G, Table 2.4*). Furthermore, plasma TMAO concentrations did not correlate with atherosclerotic lesion size (*Figure 2.3 C*).

Figure 2.5 Dietary choline and betaine supplementation 24 weeks in Ldlr$^{-/-}$ mice did not alter atherosclerotic size lesion

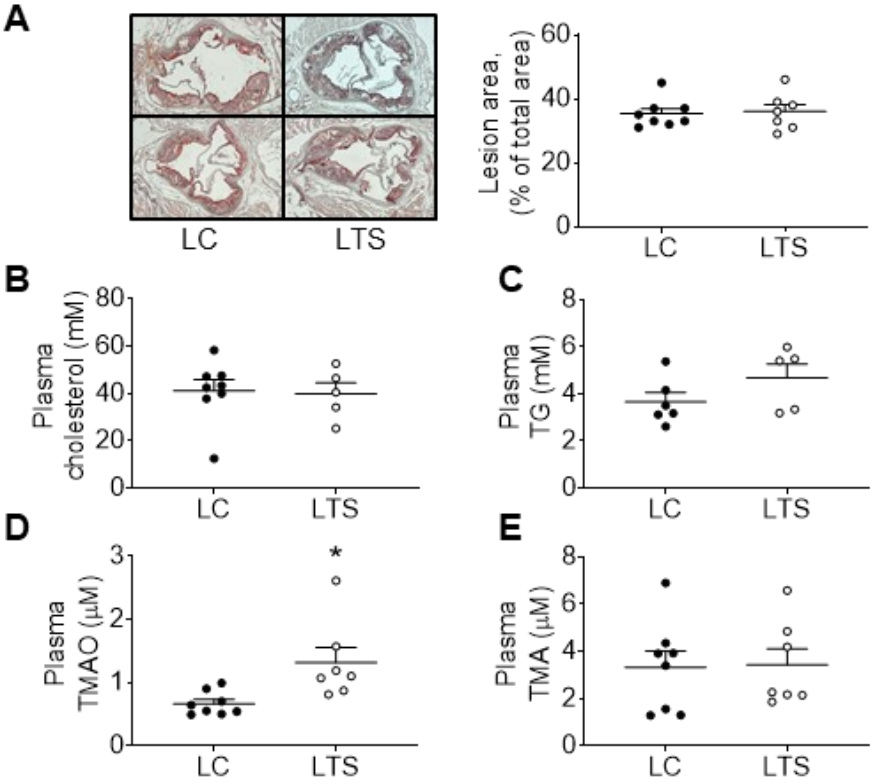


Aorta atherosclerotic lesions (A), plasma cholesterol (B), TG (C), choline (D), betaine (E), TMAO (F) and TMA (G) in 24-wk-old male *Ldlr*$^{-/-}$ mice fed with LC, LCS or LBS diet for 16 wk (Feeding Trial 3). Values are means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 12. Groups without a common letter differ, $P < 0.05$. LBS, betaine-supplemented; LC, control, LCS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

2.3.4 Feeding trial 4: dietary supplementation with TMAO for 16 wk did not alter atherosclerotic size lesion in *Ldlr*$^{-/-}$ mice

Dietary TMAO supplementation for 16 did not alter the size of atherosclerotic lesions in *Ldlr*$^{-/-}$ mice compared with the control group (*Figure 2.6 A, Table 2.4*). Furthermore, TMAO supplementation did not have any effect on body weight (*Figure 2.1 D*) or fasting plasma cholesterol and TG concentrations (*Figure 2.6 B, C, Table 2.4*). Moreover, plasma TMAO concentrations were higher (2-fold) in the LTS group than in the LC group (LTS compared with LC: 1.3 compared with 0.7 μM) (*Figure 2.6 D, Table 2.4*). Plasma TMA concentrations did not change between dietary groups (*Figure 2.6 E, Table 2.4*). Furthermore, plasma TMAO concentrations did not correlate with atherosclerotic lesion size (*Figure 2.3 D*).

Figure 2.6 Dietary TMAO supplementation for 24 weeks in Ldlr$^{-/-}$ mice did not alter atherosclerotic size lesion

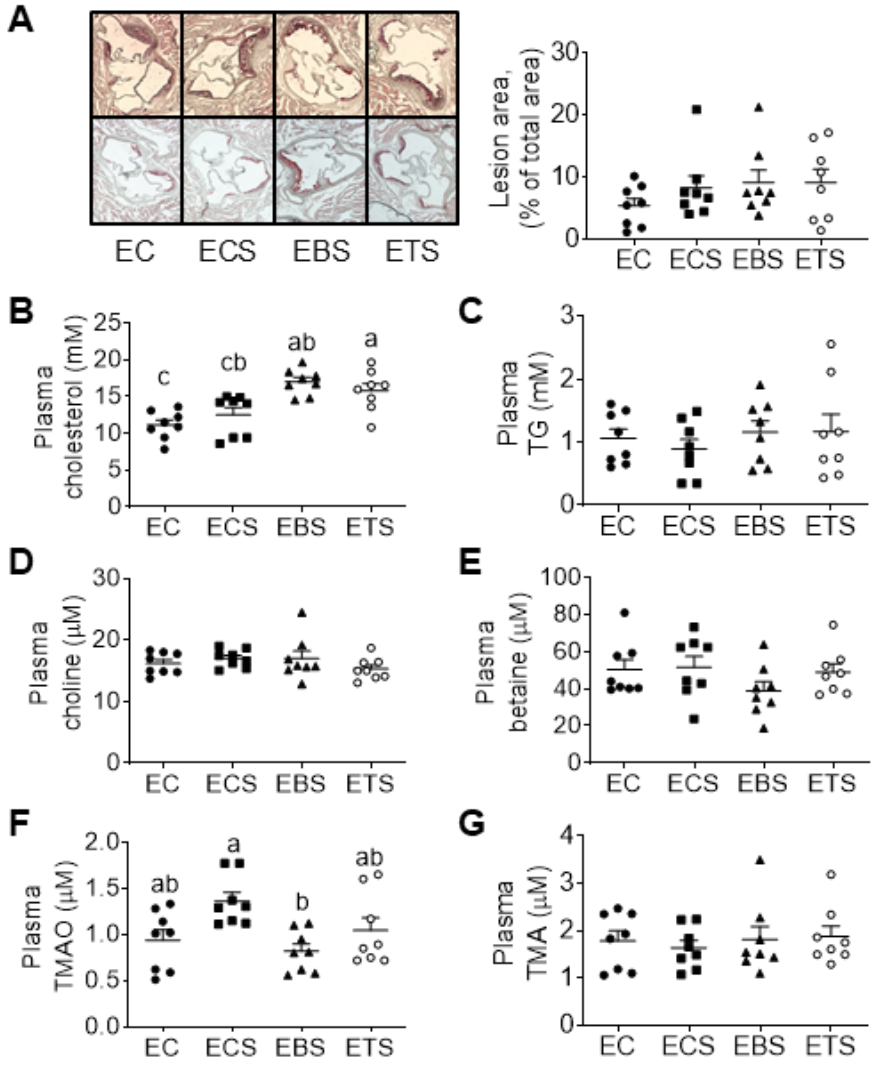


Aorta atherosclerotic lesions (A), plasma cholesterol (B), TG (C), TMAO (D), TMA (E) in 24-wk-old male *Ldlr*$^{-/-}$ mice fed with LC or LTS diet for 16 (Feeding Trial 4). Values are means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 8. *Different from control group, $P < 0.05$. LBS, betaine-supplemented; LC, control; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; LTS, TMAO-supplemented; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

2.3.5 Feeding trial: 5 dietary supplementation with choline, betaine or TMAO for 12 wk did not alter atherosclerotic size lesion in *Apoe*$^{-/-}$ mice.

Body weight did not differ between dietary groups (*Figure 2.1 E*). The size of lesions in the aortic root did not differ between dietary groups (*Figure 2.7 A, Table 2.4*). Mice that consumed EBS or ETS diets had slightly higher fasting plasma cholesterol concentrations than the EC group (*Figure 2.7 B, Table 2.4*). Plasma concentrations of TG, choline, and betaine were not different between dietary groups (*Figure 2.7 C-E, Table 2.4*). Plasma TMAO concentrations were not different in dietary- supplemented groups compared with the EC group; there was only a significant difference between the ECS and EBS groups (*Figure 2.7 F, Table 2.4*). Moreover, TMA concentrations in plasma were not different between groups (*Figure 2.7 G, Table 2.4*). Furthermore, plasma TMAO concentrations were not correlated with atherosclerotic lesion size (*Figure 2.3 E*).

Figure 2.7 Dietary choline, betaine and TMAO supplementation for 12 weeks in Apoe$^{-/-}$ mice did not alter atherosclerotic size lesion

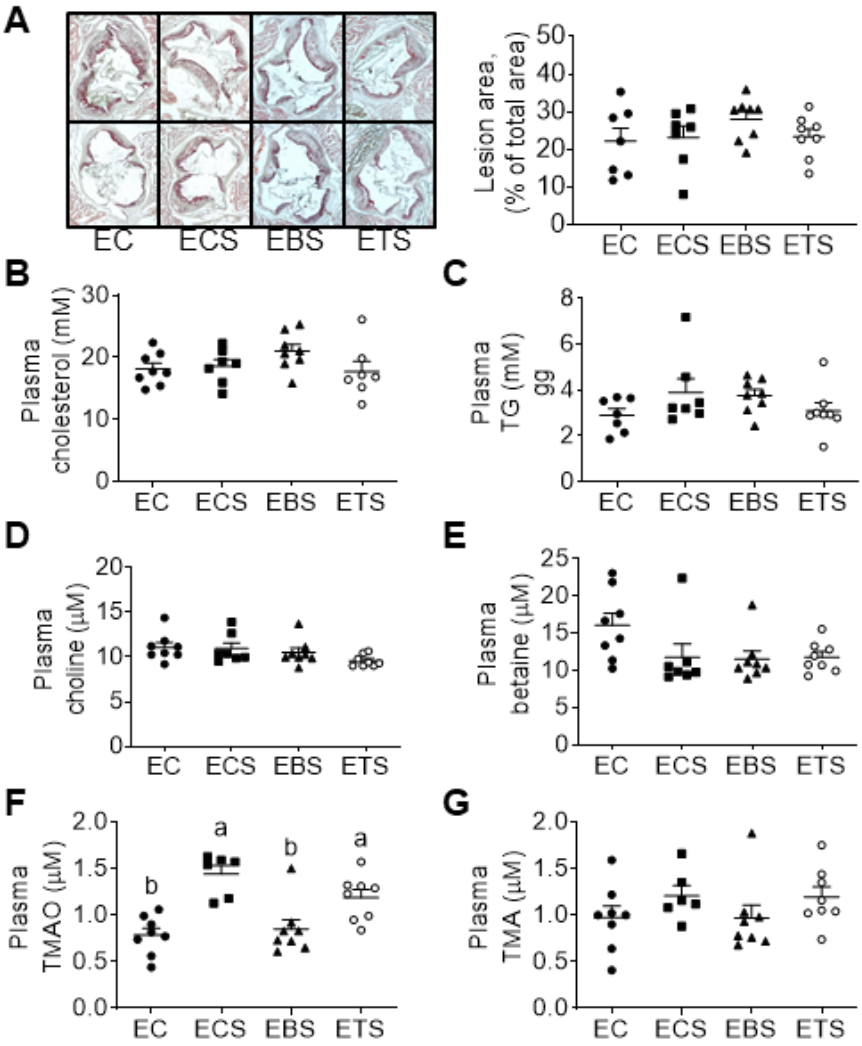


Aorta atherosclerotic lesions (A), plasma cholesterol (B), TG (C), choline (D), betaine (E), TMAO (F) and TMA (G) in 20-wk-old male *Apoe*$^{-/-}$ mice fed with EC, ECS, EBS or ETS diet for 12 wk (Feeding Trial 5). Values are means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 8. Groups without a common letter differ, $P < 0.05$. *Apoe*$^{-/-}$, Apolipoprotein E knockout; EBS, betaine-supplemented; EC, control; ECS, choline-supplemented; ETS, TMAO-supplemented; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

2.3.6 Feeding trial 6: dietary supplementation with choline, betaine or TMAO supplementation for 28 wk did not alter atherosclerotic size lesion in *Apoe*$^{-/-}$ mice.

There was no difference in atherosclerotic plaque area between dietary groups (*Figure 2.8 A, Table 2.4*). Body weight (*Figure 2.1 F*) and plasma cholesterol, TG, choline, and betaine concentrations were not different between dietary groups (*Figure 2.8 B-E, Table 2.4*). Furthermore, TMAO concentrations in plasma were significantly higher in animals fed an ECS diet (ECS compared with EC: 1.44 compared with 0.79 μM) or an ETS diet (ETS compared with EC: 1.19 compared with 0.79 μM) (*Figure 2.8 F, Table 2.4*). Concentrations in plasma of TMA did not differ between dietary groups (*Figure 2.8 G, Table 2.4*). We observed no correlation between plasma TMAO concentrations and atherosclerotic plaque area (*Figure 2.3 F*).

Figure 2.8 Dietary choline, betaine and TMAO supplementation for 28 weeks in Apoe$^{-/-}$ mice did not alter atherosclerotic size lesion



Aorta atherosclerotic lesions (A), plasma cholesterol (B), TG (C), choline (D), betaine (E), TMAO (F) and TMA (G) in 36-wk-old male *Apoe*$^{-/-}$ mice fed with EC, ECS, EBS or ETS diet for 28 wk (Feeding Trial 6). Values are means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 8. Groups without a common letter differ, $P < 0.05$. *Apoe*$^{-/-}$, Apolipoprotein E knockout; EBS, betaine-supplemented; EC, control; ECS, choline-supplemented; ETS, TMAO-supplemented; TG, triacylglycerol; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

2.4 Discussion

Choline, an essential nutrient, is required for several critical biological processes, such as synthesis of phospholipids, lipoproteins, acetylcholine, and one-carbon metabolites (Zeisel et al. 1991; Zeisel and da Costa 2009). Recently, it has been proposed that a high intake of dietary phosphatidylcholine, the major form of choline in the diet (Zeisel et al. 2003), may increase atherosclerosis risk owing to its conversion to TMAO (Tang et al. 2013; Wang et al. 2011). The present study shows that dietary supplementation of high amounts of choline or betaine does not increase atherosclerotic plaque formation in 2 atherogenic mouse models. Furthermore, providing TMAO directly in the diet did not increase atherosclerosis, despite significantly increasing plasma TMAO concentrations.

Our findings in *Ldlr*$^{-/-}$ mice show that neither short- nor long-term dietary choline or betaine supplementation increased atherosclerotic plaque size. To our knowledge, the effect of dietary choline on atherosclerosis development in *Ldlr*$^{-/-}$ mice has not been studied. Seldin et al. (Seldin et al. 2016) reported a 6-fold increase in plasma TMAO concentrations in female *Ldlr*$^{-/-}$mice supplemented with choline chloride in the drinking water for 3 wk. This treatment increased inflammation within the aorta, but overall atherosclerosis was not measured. To analyze the acute effect of TMAO on inflammation in the atherogenic process, Seldin et al. (Seldin et al. 2016) administered TMAO by intraperitoneal injection to *Ldlr*$^{-/-}$ female mice. Plasma TMAO concentration reached 100 μM 1 h after injection (Seldin et al. 2016) and was correlated with increased mRNA levels of genes (cyclooxygenase 2, IL- 6, E-selectin, and intercellular adhesion molecule 1) related to inflammation. TMAO injection also increased the nuclear abundance of total phosphorylated TNF-α in aortas (26). (Seldin et al. 2016). This study concluded that elevated TMAO concentrations can enhance production of cytokines and adhesion molecules in part by

increasing the TNF-α signaling pathway (Seldin et al. 2016). In our study, we observed only a 1.6-fold increase in plasma TMAO concentrations in the LCS group compared with controls, which did not alter the development of aortic plaques. We hypothesized that the increase in plasma TMAO in the current study, although biologically feasible from diet, was insufficient to increase atherosclerosis; therefore, in the second experiment, we fed animals a TMAO-supplemented diet and increased the feeding time. We observed higher plasma TMAO concentrations in animals fed LTS than in the LC group. However, to our surprise these mice did not have any increase in atherosclerotic plaque formation.

Because dietary supplementation of choline, betaine, or TMAO did not influence atherosclerosis formation in *Ldlr*$^{-/-}$ mice, we hypothesized that the effects of dietary choline metabolites might be dependent on the mouse model used. Several studies have assessed the atherogenic effects of dietary choline and TMAO in *Apoe*$^{-/-}$ male mice. Similar to our experimental conditions, 8-wk-old *Apoe*$^{-/-}$ mice were fed either an unpurified diet (~0.09% choline) or an unpurified diet supplemented with choline (1.0%, wt:wt) or TMAO (0.12%, wt:wt) for 12 wk (Wang et al. 2011). In that study (Wang et al. 2011), both choline and TMAO supplementation resulted in a 5-fold increase in plasma TMAO concentration and atherosclerosis in male mice. When Geng et al. (Geng et al. 2018), (40)fed 8-wk-old *Apoe*$^{-/-}$ male mice an HFD and drinking water supplemented with TMAO (1 mM) for 8 wk, they observed increased atherosclerotic lesions in the TMAO-supplemented groups; however, plasma TMAO concentrations were not reported. In contrast with those studies, we did not observe any increase in plasma TMAO concentrations or atherosclerotic plaque area in the ECS, EBS, or ETS groups after 12 wk. Furthermore, plasma TMAO concentrations were significantly greater in animals fed an ECS diet or an ETS diet for 28 wk, but atherosclerosis did not increase compared with control mice. Even though the experimental

conditions were similar, we did not observe the atherogenic effect in *Apoe*$^{-/-}$ male mice previously reported (Bennett et al. 2013; Geng et al. 2018; Tang et al. 2015; Wang et al. 2011; Zhu et al. 2016).

Consistent with our findings, 2 recent studies did not observe a positive correlation between plasma TMAO concentrations and atherosclerosis development in *Apoe*$^{-/-}$ male mice. Lindskog Jonsson et al. (Lindskog Jonsson et al. 2018) reported that choline supplementation increased plasma TMAO concentrations by 10-fold in male mice; however, no change in atherosclerosis was observed. Interestingly, TMAO treatment showed a reduction in diastolic pressure and cardiac fibrosis, despite increasing plasma TMAO concentrations (~4- to 5-fold) in spontaneously hypertensive (Huc et al. 2018). More work is required to understand the physiological consequences of TMAO in mammals.

The reasons for the discrepancy between dietary studies are unclear. The mice used in our study developed normal lesion size and progression with time; for this reason we decided to do feeding trials at early and late periods. It is possible that housing conditions across facilities are different. It has been established that TMAO production depends on TMA-lyase from bacteria in the gut (Zhu et al. 2016). In humans, it has been identified that *Firmicutes* are the main bacteria species that produce TMA from the dietary metabolites choline and l-carnitine(Cho et al. 2017). In mice, Koeth et al. (Koeth et al. 2013) showed that *Prevotella* is able to metabolize dietary choline and l-carnitine to TMA. We previously reported that *Ldlr*$^{-/-}$ mice have a high percentage of *Prevotella* (Zia et al. 2018). A recent study identified that housing factors modulate the gut microbiota composition and activity (Ericsson et al. 2018). Clearly, the relative increases in plasma TMAO concentrations after choline supplementation are not consistent between studies. It is possible that the starting age of the mouse may contribute to the differences between Wang et al. (Wang et al.

2011) (4 wk), and Lindskog Jonsson et al. (Lindskog Jonsson et al. 2018) and our study (8 wk) (Getz and Reardon 2018). Expression of hepatic FMO3, the main FMO that oxidized TMA to TMAO, decreases after 6 wk of age in male mice (Janmohamed et al. 2004). Nevertheless, the increase of plasma TMAO concentrations observed by Lindskog Jonsson et al. (Lindskog Jonsson et al. 2018) was higher than those reported by Wang et al. (Wang et al. 2011).

As in animal studies, human dietary trials have produced varying results with respect to the effect of dietary choline supplementation on plasma TMAO concentration (Cho et al. 2017; DiMarco et al. 2017; Miller et al. 2014; Missimer et al. 2018). Three studies have shown that the consumption of >2 eggs/d, rich in phosphatidylcholine, did not change plasma TMAO concentrations (Cho et al. 2017; DiMarco et al. 2017; Missimer et al. 2018). However, 1 study reported an increase of plasma TMAO concentrations after consuming >2 egg yolks in a meal (Miller et al. 2014). Diverse systematic reviews and meta-analysis studies have shown that egg consumption is not associated with heart disease risk or mortality (Richard, Cristall, et al. 2017; Shin et al. 2013; Xu et al. 2018). To date, as far as we know there is no study that demonstrates a positive association between estimated choline intake and CVD risk. A cohort study and a systematic review concluded that choline intake was not significantly associated with CVD risk (Meyer and Shea 2017; Nagata et al. 2015).

In conclusion, our study used 2 atherogenic mouse models and showed that dietary choline or TMAO supplementation does not induce atherosclerosis development, despite increasing plasma TMAO concentrations.

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Chapter 3: Dietary phosphatidylcholine supplementation reduces atherosclerosis in *Ldlr*$^{-/-}$ male mice

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3.1 Introduction

Choline is an essential nutrient that plays important biological roles, such as being a source of methyl groups, a precursor for the biosynthesis of phospholipids and acetylcholine, and a regulator of lipoprotein metabolism (Zeisel et al. 1991; Zeisel and da Costa 2009). Excess of dietary choline is metabolized by the gut microbiota in the intestine and cecum to trimethylamine (TMA) (de la Huerga and Popper 1951; Romano et al. 2015; Zeisel 1981). TMA is absorbed by enterocytes and is delivered to the liver through the portal vein. In the liver, TMA is oxidized to trimethylamine N-oxide (TMAO) by the flavin containing monooxygenase (FMO) enzymes (Lang et al. 1998; Wang et al. 2011; Zhang et al. 1999). In the last decade, epidemiological and animal studies suggested that high choline intake may increase cardiovascular disease risk by elevating plasma TMAO levels (Gregory et al. 2015; Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; Lever et al. 2014; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; Wilson Tang et al. 2015; Tang et al. 2015b; Wang et al. 2011, 2014; Zhu et al. 2016). In contrast, two recent animal studies did not observe a positive correlation between plasma TMAO levels and cardiovascular disease (CVD) (Huc et al. 2018; Lindskog Jonsson et al. 2018). Huc et al. reported that low-dose TMAO treatment reduced diastolic dysfunction and heart fibrosis in spontaneously hypertensive rats (Huc et al. 2018). Lindskog Jonsson et al. observed that choline supplementation did not affect atherosclerotic lesion size nor plasma cholesterol levels in *Apoe*$^{-/-}$ mice (Lindskog Jonsson et al. 2018). In concordance, we previously showed that dietary TMAO and choline supplementation did not increase atherosclerosis development in two atherogenic mouse model (*Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice), despite an elevation of plasma TMAO levels (Aldana-Hernández et al. 2020).

To date, free-choline (choline bitartrate or choline chloride) (Aldana-Hernández et al. 2020; Gregory et al. 2015; Lindskog Jonsson et al. 2018; Wang et al. 2011; Zhu et al. 2016), but not phosphatidylcholine (PC), has been used in animal studies investigating the association of dietary choline with atherosclerosis. Two major moieties of choline in the diet of the North American population are PC and free-choline (Yonemori et al. 2013). Koeth et al suggested that the consumption of meat and eggs, major sources of dietary PC, increases CVD risk (Koeth et al. 2013). However, PC is also found in vegetables like soybeans, spinach, nuts and seeds (Patterson et al. 2008a), which were not taken into account. Interestingly, dietary PC can also have beneficial effects on metabolism. Several studies have shown that dietary PC supplementation can lower plasma cholesterol by reducing fractional absorption of cholesterol, and it can enhance immune development and function in animal trials (Dellschaft et al. 2018; Lewis et al. 2017; Lewis, Richard, et al. 2015; Richard, Lewis, et al. 2017).

It is well known that PC biosynthesis plays a critical role in lipoprotein assembly and secretion (Cole, Vance, and Vance 2012). Rats fed a choline deficient diet for 3 days have reduced plasma very low-density lipoprotein (VLDL), but not plasma high-density lipoprotein (HDL), due to impaired VLDL secretion (Yao and Vance 1990). Liver specific CTα (the rate limiting enzyme in the Kennedy-PC biosynthesis pathway) knockout mice showed a reduction in plasma triacylglycerol (TG), cholesterol and PC (Jacobs et al. 2004). In these mice, both VLDL and HDL secretion were diminished. Another PC biosynthesis pathway in the liver is through three sequential methylation reactions of phosphatidylethanolamine (PE) to PC, mediated by phosphatidylethanolamine N-methyltransferase (PEMT) (Bremer and Greenberg 1960; van der Veen, Kennelly, et al. 2017). *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$ mice fed a high fat/high cholesterol diet had lower atherosclerosis size lesion than *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$ mice (Zhao et al. 2009). The atheroprotective effect

was due to a reduction in plasma VLDL/IDL/LDL; as HDL levels were not altered (Zhao et al. 2009). Hepatic VLDL secretion was reduced in double *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice (Zhao et al. 2009). Interestingly, in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice the PC/PE ratio of VLDL particles was lower compared to *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$, which resulted in increased clearance of VLDL particles from the circulation (Zhao et al. 2009).

The aim of this study was to investigate the effect of dietary PC supplementation on atherosclerosis development in male *Ldlr* $^{-/-}$ mice. We hypothesized that dietary choline supplementation (either as free choline or PC) would not enhance atherosclerotic plaque formation in male *Ldlr* $^{-/-}$ mice.

3.2 Materials and methods

3.2.1 Animal handling

All animal experiments were approved by the Institutional Animal Care Committee at the University of Alberta (AUP00000175), according with guidelines of the Canadian Council on Animal Care. Before the experiment, male C57Bl/6J *Ldlr*$^{-/-}$ mice were fed an unpurified standard diet (PicoLab® Laboratory Rodent Diet 5L0D*). At 8-10 wk of age, mice were given free access to one of the three experimental diets for 12 wk. Mice weights were monitored throughout the study to ensure wellbeing, but only the initial and final weight at dissection were considered for data analysis. At the end of the experiment, mice were fasted for 12 h and euthanized by cardiac puncture. Blood was collected in EDTA-containing tubes and centrifuged at 5000 rpm at 4°C for 10 minutes to separate plasma. The plasma was stored at -80°C. Tissues were collected aseptically, weighed, snap frozen in liquid nitrogen, and stored at −80 °C until further analysis. Gonadal fat pad and interscapular brown adipose tissue (BAT) pads were collected and weighed. Formalin-fixed paraffin- embedded livers were sectioned for histological analysis using hematoxylin-eosin (H&E) staining.

3.2.2 Diets

Mice were randomized to one of the three high-fat diet (HFD: 40% fat of calories and 0.5% wt/wt of cholesterol to induce atherosclerosis in the *Ldlr*$^{-/-}$ mice): control (CON, *n*=21) (0.1% choline wt/wt), choline-supplemented (CS, *n*=21) (0.4% choline wt/wt), or PC-supplemented (PCS, *n*=24) (0.1% choline and 0.3% of choline from PC wt/wt) (*Table 3.1* and *3.2*). Because the PC used contains fatty acids, the diets were matched for lipid content and composition to provide 40% of calories from lipids. Choline bitartrate (C1629) was purchased from Sigma-Aldrich®. PC used was Soy Lecithin Granules, which was purchase from GNC (code 005648).

Table 3.1 Composition of experimental diets [1]

Diet ingredients	CON	CS	PCS
Total Protein (g (%, kcal))	272 (23)	272 (23)	272 (23)
Casein[2] (g)	270	270	270
L-cysteine[4] (g)	1.8	1.8	1.8
Total Carbohydrates (g (%, kcal))	446 (38)	446 (38)	446 (38)
Sucrose[3] (g)	195.35	195.35	195.35
Corn starch[2] (g)	170.65	170.65	170.65
Cellulose[2] (g)	80	80	80
Inositol[4] (g)	6.3	6.3	6.3
Total Fat (g (%, kcal))	205 (39)	205 (39)	213 (40)
Lard[3] (g)	155	155	127
Crisco Vegetable oil[3] (g)	32	32	23
Mazola corn oil[3] (g)	10	10	10
Cholesterol[4] (g)	5	5	5
DHAsco[5] (g)	1.5	1.5	1.5
ARAsco[5] (g)	1.5	1.5	1.5
Total energy (kcal)	4,716	4,716	4,788
Vitamin mix (AIN-93) [2,6] (g)	19	19	19
Bernhart–Tomerelli mineral mix[2,7] (g)	50	50	50
Calcium phosphate dibasic[4] (g)	3.4	3.4	3.4
Choline bitartrate[4] (g)	2.5	10	2.5
PC (soy lecithin)[8] (g)	0	0	90 (48 g of fat)

[1]Choline and PC supplements were mixed to obtain 1 kg of the high-fat diet.
[2]Ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA).
[3]Ingredients were purchased from Safeway (Edmonton, AB, Canada).
[4]Ingredients from (Sigma-Aldrich), choline bitartrate (C1629), betaine BioUltra, ≥99.0% (NT) (CAS 107-43-7) and TMAO 95% (CAS 1184-78-7).
[5]Oils were donated by DSM (Nutritional Products, Columbia, MD, USA).
[6]AIN-93-VX vitamin mix (Reeves 1997)
[7]Bernhart-Tomarelli mineral mixture (Bernhart and Tomarelli 1966)
[8]Soy Lecithin Granules from GNC, code 005648. The soy lecithin contained 23% (w/w) PC, equivalent to 3.2% (w/w) choline

Table 3.2 Fatty acid composition of experimental diets[1]

Fatty Acid	CON	CS	PCS
	g/100 g of total fatty acids		
C16:0	21.4	21.4	20.3
C16:1n9	1.8	1.8	1.4
C18:0	12.5	12.5	10.6
C18:1n9	37.8	37.8	33.6
C18:2n6	23.4	23.4	30.2
C20:0	0.3	0.3	0.3
C18:3n3 (ALA)	1.7	1.7	2.7
C20:3n6	0.5	0.5	0.4
C20:4n6 (AA)	0.3	0.3	0.3
C22:6n3 (DHA)	0.3	0.3	0.3
Other fatty acids	1.3	1.3	1.0
Total SFA	35.1	35.1	31.8
Total PUFA	25.9	25.9	33.5
Total n-6	23.9	23.9	30.6
Total n-3	2.4	2.4	3.3
Total MUFA	39.0	39.0	34.6
n-6/n-3	9.8	9.8	9.3
PUFA/SFA	0.7	0.7	1.1

[1]Analysis of the fat mixture added to experimental diets determined by gas liquid chromatography; AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids;

[2]other fatty acids refer to fatty acids that contributed for less than 0.2% in the diet which included trace of 10:0, 12:0, 14:0, 18:1c11, 20:1, 20:5n-3, 22:0.

3.2.3 Analysis of atherosclerosis

Hearts were perfused through the left ventricle with PBS containing 5mM EDTA. Hearts were then removed and incubated in Krebs-Henseleit buffer for 30 mins, fixed overnight in 10% phosphate buffered formalin, and cut cross-sectional to the aortic root. This section was incubated in 30% sucrose solution overnight and then embedded in optimal cutting temperature compound (Cryomatrix Thermo Scientific) and stored at -20°C. The aortic root was cryosectioned (10 μm thick) and stained with Oil Red O and Mayer's Hematoxylin (Sigma-Aldrich) (Zhao et al. 2009). The area of atherosclerotic plaque was quantified and calculated as Oil Red O-positive area as percentage of total cross aortic surface area by using ImageJ from the National Institutes of Health in 9 cross-sections (3 sets of 3 consecutive sections, 200 μm apart) over 520 μm aortic root.

3.2.4 Metabolite analysis

Plasma total cholesterol and TG levels were measured by commercially available kits from Wako (Catalog No. 999-02601) and SEKISUI Diagnostics (Catalog No. 236-60), respectively. Plasma lipoprotein fractions were separated by fast protein liquid chromatography (Vance, Weinstein, and Steinberg 1984). Plasma choline, betaine, TMA and TMAO were quantified by hydrophilic interaction liquid chromatography–tandem mass spectrometry as previously described (Mi et al. 2017; Xiong et al. 2012; Y. Y. Zhao, Xiong, and Curtis 2011). For the liver the mass of TG, total cholesterol, cholesterol ester, and total phospholipid/lysophosplipid were measured by gas-liquid chromatography (Kuksis and Myher 1989). Hepatic PC and PE were isolated by thin-layer chromatography and quantified using a phosphorous assay and molar ratio of PC/PE was calculated (Bligh and Dyer 1959; Rouser, Siakotos, and Fleischer 1966).

3.2.5 VLDL secretion

After a 12 h fast, mice were injected intra-peritoneally with Poloxamer 407 (1 g/Kg) to inhibit lipoprotein lipase (Millar et al. 2005). Blood samples were collected at 0, 1, 2, 3, and 4 h for lipid analysis.

3.2.6 In vivo immune spleen and peripheral blood cells phenotypes

Immune cells from spleen were isolated as previously described (Field et al. 1990). Briefly, spleens were disrupted and cell suspension was obtained by passing the cells through a nylon mesh screen in sterile Krebs-Ringer HEPES buffer with bovine serum albumin (5 g/L, from Sigma-Aldrich CA). For the isolation of circulating immune cells, blood was lysed with 1% (v/v) BD Pharm Lyse buffer from Bioscience (Catalog No. 555899) to remove erythrocytes. Then the leucocytes were washed three times with PBS containing 5% (v/v) heat-inactivated fetal calf serum, and 1% antibiotic/antimycotic. Immune cells were counted on a haemocytometer with trypan blue dye from Sigma-Aldrich (Catalog No. T8154) and diluted to $1.25x10^6$ cells/mL. Immune cell subsets were identified by immunofluorescence assay, as described previously (Field et al. 1990, 2000). Briefly, immune cells were incubated with pre-labelled monoclonal antibodies for 30 min at 4ºC. Antibodies (purchase from Biolegend; San Diego, CA, USA) were added in combination of four-color flow cytometry to determine the phenotypes of immune cells. For surface molecules on immune cells from spleen tissue, the combinations used were as follow: CD4/CD3/CD19/CD11b, CD4/CD25/CD80, CD68/CD36/CD54/CD284. For circulating leukocytes from blood two combinations were used: CD4/CD25/CD80, CD68/CD36/CD54/CD284. Cells were washed and fixed with paraformaldehyde (1 % w/v in PBS). Within 72 h, samples were acquired by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) according to the relative

fluorescence intensity determined using Kaluza Software (Beckman Coulter, Mississauga, ON, Canada).

Circulating chemokines and cytokines levels were measured by a Mouse Cytokine Array / Chemokine Array 31-Plex (MD31) (Eve Technologies Corporation; Calgary, AB).

3.2.7 Western Blots Analysis.

Western blot analyses were performed as previously described (Zhao et al. 2009). Plasma apolipoproteins were identified with commercially available antibodies: APOB (from Millipore, Catalog No. AB742), APOE, and APOA1 from Biodesign (Catalog No. K7410G, and K23001R, respectively). Immunoreactive proteins were visualized with ECL from GE Healthcare (Catalog No. 45002401 PM) using a charge coupled device-camera and Fluor-S Multi-Imager system (Bio-rad Laboratories, CA, USA). Ponceau staining was used as loading control. The amount of protein was quantified using Image Lab software from Bio-Rad.

3.2.8 Real-time quantitative PCR

Total RNA from aorta and liver was isolated with TRIzol reagent from Invitrogen (Catalog No. 15596018). The total RNA was treated with DNase I from Invitrogen (Catalog No. 18068-015) to degrade genomic DNA. Then RNA was reverse transcribed using oligo(dT)12–18 primers (*Table 3.3*) and Superscript II reverse transcriptase from Invitrogen (Catalog No. 18418-012 and 18064-014) according to the manufacturer's instructions. Real-time quantitative PCR was run with Power SYBR™ Green PCR Master Mix (Catalog No. 4367659) in a Step One Plus qPCR system. The data was analyzed with StepOne Software v2.2.2 (Applied Biosystems) and mRNA levels were normalized to cyclophilin and *Gapdh* (liver and whole aorta, respectively) mRNA.

Table 3.3 Primers for mRNA analysis[1]

Gene	Forward 5′-3	Reverse 5′-3′
Abca1	AGT TTC TGC CCT CTG TGG TC	GGG TCG GGA GAT GAG ATG T
Abcb11 (Bsep)	CAG TGG GTG TGG TAA AAG CA	TGC TGT CGT GAC CAT CTA TCA
Abcb4 (Mdr2)	TTG TCA ATG CTA AAT CCA GGA A	TTG GAT ATA GGC AGC CAC AAG
Abcg1	CCT CTC GCA CGG CTC TCA	TGA ACT GCC CTA CCT ACC ACA A
Abcg5	CTG CTC GCC TAC GTG CTA	ATC TGG CAA CTT CAG GAT ACA A
Acat1	TAC GCT TTG GTG ACA GGA TG	TCC AGG TCC TGT AGT AGT TGG AG
Acat2	CCA GCT TCG GAG GAG AGA A	AGT CTG GGG TTC CGT GTG T
Cd36	TGG CTA AAT GAG ACT GGG ACC	ACA TCA CCA CTC CAA TCC CAA G
Cd45	CTG ACA ATC CCA CAC TCA CG	TCC CCG GTA CAG TCC TCT C
Cd68	GCG GCT CCC TGT GTG TCT GAT	GGG CCT GTG GCT GGT CGT AG
Cyp27a1	CTT TCC TGA GCT GCT TTT GG	CAC CAG TCA CTT CCT TGT GC
Cyp7a1	ACA CCA TTC CTG CAA CCT TC	TCT TGG CCA GCA CTC TGT AA
F4/80	CCC TCG GGC TGT GAG ATT GTG	TGG CCA AGG CAA GAC ATA CCA G
Fmo1	ACA TTA CCA CCG CCA AGT GT	TGC AGT AGC ACA AGC CAA AC
Fmo2	CTG GAG AAG CCA ACC CTT G	CCT ACA CGG TTC AAG ATC CAA
Fmo3	GGG AAC TCA GGC TGT GAC AT	GAC TCG ACT CAT CAC CCA AGA
Fmo5	GAT TAG CCA CAC AGC CAA GC	CCG AAG GCA AGC TAC ACA A
Icam-1	GCT ACC ATC ACC GTG TAT TCG	AGG TCC TTG CCT ACT TGC TG
Il-10	GCT GGA CAA CAT ACT GCT AAC	CCG CAT CCT GAG GGT CTT C
Il-1b	GAA GTT GAC GGA CCC CAA AA	CCA CGG GAA AGA CAC AGG TAG
Il-2	GCT GTT GAT GGA CCT ACA GGA	TTC AAT TCT GTG GCC TGC TT
Il-4	CAT CGG CAT TTT GAA CGA G	CGA GCT CAC TCT CTG TGG TG
Il-6	ACA AAG CCA GAG TCC TTC AGA	TGG TCC TTA GCC ACT CCT TC
Ifng	ATC TGG AGG AAC TGG CAA AA	TTC AAG ACT TCA AAG AGT CTG AGG
Lcat	GGC TGA ACT CAG TAA CCA CAC A	TTG GCT TCT AGC CGA TTC C
Olr1	CCT GCT GCT ATG ACT CTG GTC	GAG GTC AGA TAC CTG GCG TAA
Msr1	CCA AAC GCA CTC CCC TTA C	CAC TGG AGG TGG TCC AGA AG
Scarb1	GCC CAT CAT CTG CCA ACT	TCC TGG GAG CCC TTT TTA CT
Tnfa	GTC TAC TGA ACT TCG GGG TGA	CAC CAC TTG GTG GTT TGC TAC GAC
Vcam-1	TCT TAC CTG TGC GCT GTG AC	ACT GGA TCT TCA GGG AAT GAG T
Vldlr	ACT GAT GCG GCT TCT AAG AC	CAG TAA ACA AAG CCC GAC AAC C
Cyclophilin	TCC AAA GAC AGC AGA AAA CTT TCG	TCT TCT TGC TGG TCT TGC CAT TCC
Gapdh	GGG TTC CTA TAA ATA CGG ACT GC	CCA TTT TGT CTA CGG GAC GA

[1] Primers were purchased from Integrated DNA Technologies, Inc.

3.2.9 Statistical analysis

Graphs showed individual data to highlight the natural variability of biological experiments. To compare the effect of diets, Graph Pad Prism 8 was used for statistical analysis of data. Results are reported as mean ± SEM. Data was tested for normal distribution by Shapiro-Wilk normality test. Data that were not normally distributed, were Log-transformed. Log-transformed data are reported as median and 95% CI. The effects of dietary intervention were analyzed using one way- ANOVA followed by a Tukey's post-hoc test. Significance was set at $p<0.05$ in all analyses. Groups that do not share a letter are significantly different.

3.3 Results

3.3.1 Dietary PC supplementation reduced atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice

The initial body weight of the mice was similar among the dietary groups (*Table 3.4*). At the end of the dietary challenge, body weight was significantly higher in PCS group than CON and CS groups (*Table 3.4*). BAT (%, weight/body weight) was significant higher in PCS group compared to CON group (*Table 3.4*). Meanwhile, WAT and liver (% weight/body weight) were similar among the groups (*Table 3.4*). Fasting glucose levels were not changed by dietary treatments (*Table 3.4*). Atherosclerotic plaque size was lower in PCS group as compared to both the CON and CS groups (*Figure 3.1 A, B*). Meanwhile, plasma choline concentration did not differ between groups (*Figure 3.1 C*). The CS group had lower plasma betaine concentrations compared to the CON (*Figure 3.1 D*) but not the PC group. Plasma TMAO and TMA concentrations were higher in the PCS group compared to the CON and CS groups. (*Figure 3.1 E, F*). However, mRNA levels of hepatic *Fmo*s were not different among dietary groups (*Table 3.5*). Plasma cholesterol concentrations were higher in the CS group compared to the PCS group but not compared to the

CON group (*Figure 3.1 G*). Moreover, plasma TG concentrations were slightly higher in the CS group (*Figure 3.1 H*).

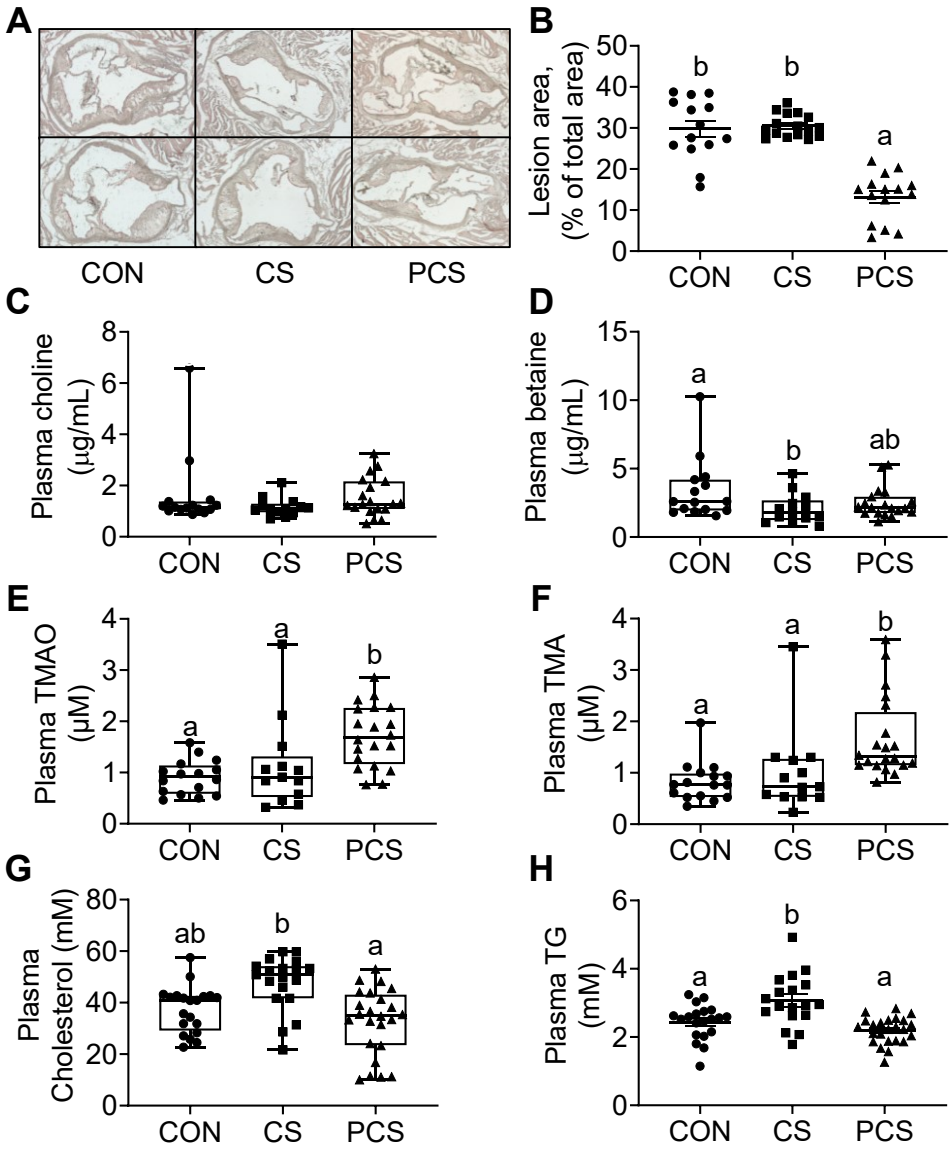
Table 3.4 Anthropometric data from Ldlr$^{-/-}$ mice fed with experimental diets for 12 wk

Variable	CON (*n* = 21)	CS (*n* = 21)	PCS (*n* = 24)	*p* Value
Initial BW (g)	25.0 [23.7-25.5]	23.6 [23.1-25.1]	24.8 [23.6-25.3]	0.733
Final BW (g)	35.7 ± 5.5[a]	36.7 ± 3.4[a]	40.9 ± 5.9[b]	0.002
Gonadal fat pad weight/Final BW (%)	5.2 [4.6-5.8]	5.4 [5.0-5.8]	5.9 [4.9-6.0]	0.665
BAT weight/Final BW (%)	0.4 ± 0.13[a]	0.5 ± 0.09[ab]	0.6 ± 0.15[b]	0.003
Liver weight/Final BW (%)	3.9 ± 0.34	3.8 ± 0.23	3.7 ± 0.70	0.503
Fasting blood glucose (mmol/L),	6.0 ± 1.1	6.3 ± 1.1	6.5 ± 1.4	0.305

Values are presented as means ± SD or medians [95% CIs]. *p* value of the main effect of diet analyzed by one-way ANOVA followed by a Tukey's post-hoc test. Multiple comparisons between diet. Within experiment, labeled values in a row without a common superscript letter differ. Significance was set at $p<0.05$ in all analyses $p < 0.05$.

BAT, brown adipose tissue; BW, body weight; CON, control; CS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; PCS, phosphatidylcholine-supplemented.

Figure 3.1 Atherosclerotic lesion size and plasma choline-metabolites and lipids in Ldlr$^{-/-}$ mice



Atherosclerotic lesions in the aortic root (A, B), plasma choline (C), betaine (D), TMAO (E), TMA (F), cholesterol (G), and TG (H) levels in 20-22-wk-old male *Ldlr*$^{-/-}$ mice fed with CON, CS or PCS diets for 12 wk. Values are reported as means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 13-24. Groups without a common letter differ, $p<0.05$. CON, control; CS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; PCS, phosphatidylcholine-supplemented; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

Table 3.5 Hepatic mRNA levels from Ldlr$^{-/-}$ mice fed with experimental diets for 12 wk

Genes	CON (*n*=6)	CS (*n*=5)	PCS (*n*=6)	*p* Value
TMAO production				
Fmo1	1.00 ± 0.34	1.18 ± 0.26	0.78 ± 0.17	0.075
Fmo2	1.00 ± 0.42	1.04 ± 0.21	0.75 ± 0.13	0.210
Fmo3	1.00 ± 0.15	1.24 ± 0.36	1.04 ± 0.12	0.205
Fmo5	1.00 ± 0.29	1.50 ± 0.53	1.07 ± 0.27	0.091
Cholesterol uptake				
Scarb1	1.00 ± 0.28^{a}	2.57 ± 1.20^{b}	1.17 ± 0.64^{a}	0.009
Vldlr	1.00 ± 0.47	1.13 ± 0.81	0.41 ± 0.24	0.088
Cholesterol efflux				
Abca1	0.94 [0.72-1.28]^{ab}	2.04 [1.51-2.25]^{a}	1.02 [0.42-1.29]^{b}	0.009
Abcg5	1.00 ± 0.25^{ab}	1.24 ± 0.48^{a}	0.75 ± 0.17^{b}	0.036
Abcg1	1.00 ± 0.30^{ab}	1.39 ± 0.73^{a}	0.40 ± 0.15^{b}	0.008
Abcb4 (Mdr2)	0.83 ± 0.45	1.37 ± 0.25	1.02 ± 0.45	0.123
Abcb11 (Bsep)	1.00 ± 0.17^{a}	2.11 ± 0.68^{b}	1.32 ± 0.76^{ab}	0.023
Cholesterol metabolism				
Acat1	1.00 ± 0.37^{ab}	1.23 ± 0.49^{a}	0.51 ± 0.10^{b}	0.010
Acat2	1.00 ± 0.20	1.28 ± 0.21	0.96 ± 0.33	0.124
Lcat	1.00 ± 0.33	1.60 ± 0.51	1.28 ± 0.63	0.179
Bile acid metabolism				
Cyp7a1	1.00 ± 0.21^{a}	0.65 ± 0.31^{ab}	0.49 ± 0.41^{b}	0.044
Cyp27a1	1.00 ± 0.27	1.25 ± 0.43	0.76 ± 0.44	0.146

Values are presented as means ± SD or medians [95% CIs]. *p* value of the main effect of diet analyzed by one-way ANOVA followed by a Tukey's post-hoc test. Multiple comparisons between diet. Within experiment, labeled values in a row without a common superscript letter differ. Significance was set at $p<0.05$ in all analyses $p < 0.05$.
CON, control; CS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; PCS, phosphatidylcholine-supplemented.

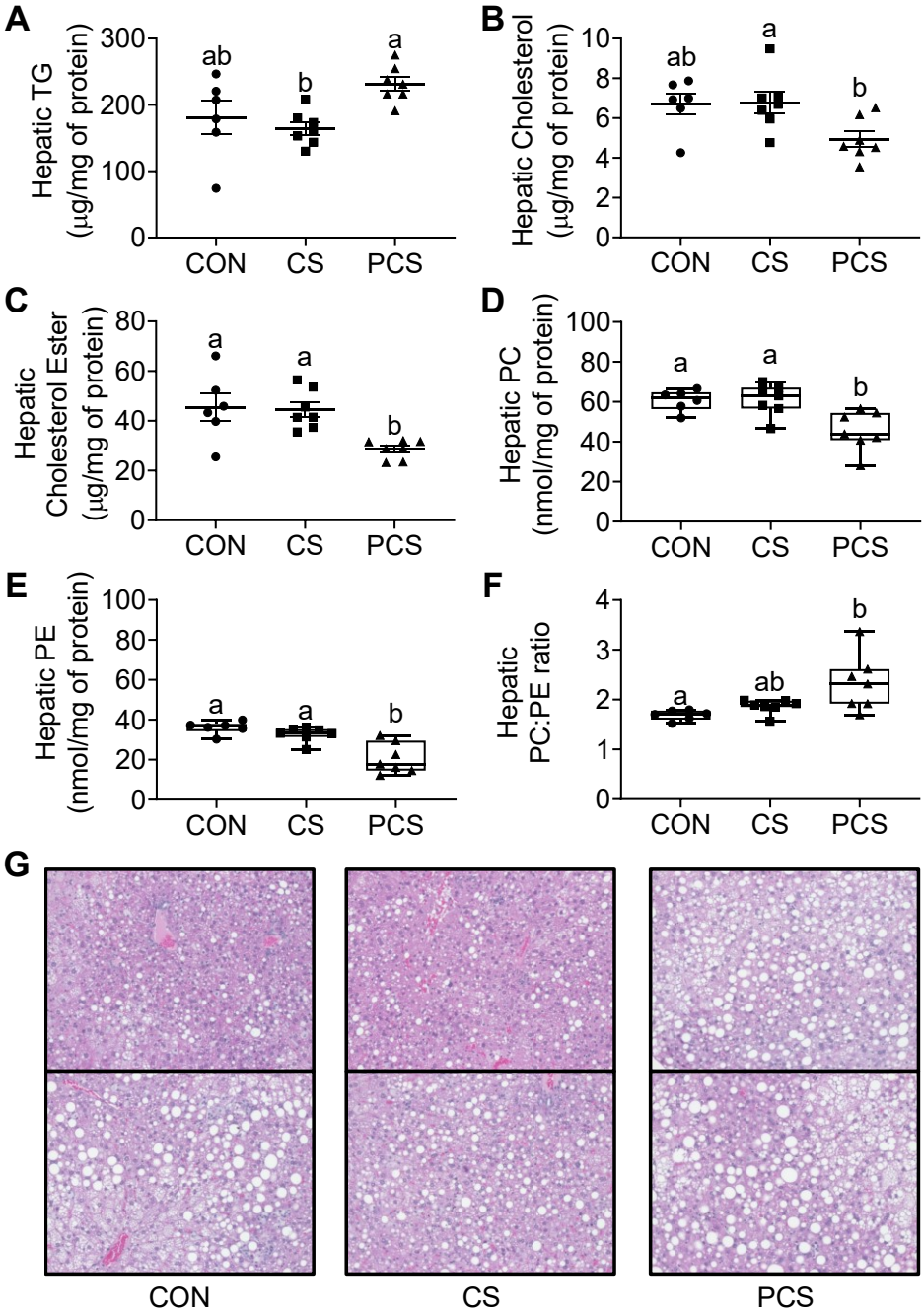
3.3.2 PCS diet reduced VLDL-C and increased HDL-C fraction in plasma

Hepatic PC and PE levels were lower in the PCS as compared to the CON and CS groups (*Figure 3.2 D, E*). However, the PC/PE ratio was higher in the PC group compared to CON group (*Figure 3.2 F*). The total phospholipid/lysophospholipid content in the liver did not change among the groups (CON 27.23± 6.63, n=6; CS 29.10 ± 3.66, n=7; and PCS 30.23 ± 6.01 μg/mg of protein, n=7; mean ± SD). Separation of lipoprotein fractions revealed that the PCS group had lower plasma VLDL-C compared to both the CON and CS groups (*Figure 3.3 A*). Plasma VLDL-TG fraction was slightly lower in PCS group and higher in CS group (*Figure 3.3 B*). Assessment of VLDL secretion revealed that only 4 h after injection with Poloxamer 407, the PCS and CS groups had higher plasma TG levels compared to the CON group (*Figure 3.3 E*). Meanwhile, 4 h after injection with Poloxamer 407, the CS group had significantly higher plasma VLDL-C (44.58 mM ± 1.43, $n = 9$) compared to CON (33.76 mM ± 1.75, $n = 9$) and PCS (36.8 mM ± 1.91, $n = 11$). We observed that plasma APOB48 and APOA1 were lower in mice fed a PCS diet compared to CON and CS groups (*Figure 3.3 C, D*), whereas plasma APOB100, and APOE did not change among dietary groups (*Figure 3.3 C, D*). In PCS group hepatic TG levels were higher compared to CS but not to CON (*Figure 3.2 A, B*). As well, the H&E staining in the liver showed that lipid droplets were more abundant in the PCS group (*Figure 3.2 G*). The reduction of plasma VLDL and APOB48 in PCS group might indicate that the VLDL clearance is more efficient.

We observed that plasma HDL-C fraction was higher in the PCS group compared to both the CON and CS groups (*Figure 3.3 A*). In the PCS group, hepatic total cholesterol levels were lower compared to CS but not to CON (*Figure 3.2 A, B, G*). Meanwhile, hepatic cholesterol ester levels were lower in PCS group than CON and CS groups (*Figure 3.2 C*). We hypothesized that the PCS diet improve the clearance of cholesterol-containing lipoprotein from the atherosclerotic plaque.

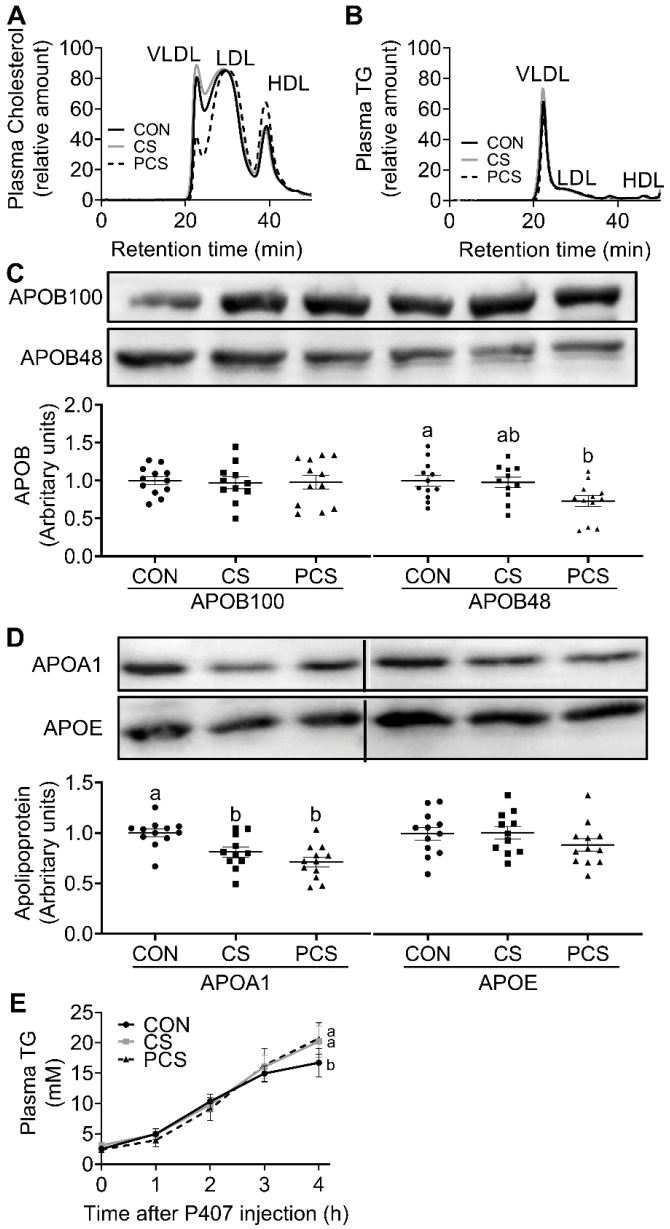
Therefore, we measured mRNA levels of genes related to cholesterol uptake-efflux and metabolism in liver (*Table 3.5*). For cholesterol uptake, we observed that *Scarb1* mRNA was higher in CS group compared to CON and PCS groups. However, *Vldlr* mRNA were similar among dietary groups. For genes related to cholesterol efflux, the relative mRNA levels of *Abca1*, *Abcg5*, and *Abcg1* were lower in PCS group compared to CS group. For genes related to cholesterol esterification, *Acat1* mRNA levels were lower in PCS group compared to CS group. However, *Lcat* mRNA did not change among the dietary groups. *Cyp7a1* was lower in PCS group compared to CON group. Meanwhile *Cyp27a1* levels were similar among dietary groups. For bile acid secretion, *Abcb11* (*Bsep*) levels were higher in CS group compared to CON group.

Figure 3.2 Hepatic lipids in Ldlr$^{-/-}$mice



Hepatic TG (A), cholesterol (B) cholesterol ester (C), PC (D), PE (E), PC/PE ratio (F), and H&E staining in livers (G) from 20-22-wk-old male *Ldlr*$^{-/-}$ mice fed with CON, CS or PCS diet for 12 wk. Values are reported as means ± SEMs or median [95% CI] in Whisker's plots for normalized data, n = 6-7. Groups without a common letter differ, $p<0.05$. CON, control; CS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout and PCS, phosphatidylcholine-supplemented.

Figure 3.3 Fasted plasma lipoprotein fractions, apolipoprotein western blots and quantification, and VLDL secretion in in Ldlr$^{-/-}$ mice



Lipoprotein fraction of plasma cholesterol (A) and TG (B), plasma APOB48 and APOB100 (C), APOA1 and APOE (D), and plasma VLDL-TG secretion from liver (at 0h, CON 2.5^{b} mM ± 0.11, n = 9; CS 3.1^{a} mM ± 0.15, n = 9; PCS 2.4^{b} mM ± 0.08, n = 9; $p < 0.0001$ and at 4 h, CON 16.7^{b} mM ± 0.78, n = 9; CS 20.2^{a} mM ± 0.86, n = 9; PCS 20.7^{a} mM ± 0.86, n = 9; $p < 0.0001$) (E) in 20-22-wk-old male *Ldlr*$^{-/-}$ mice fed with CON, CS or PCS diet for 12 wk. Values are reported as means ± SEMs, n = 9-12. Groups without a common letter differ, $p<0.05$. CON, control; CS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout and PCS, phosphatidylcholine-supplemented; P407, Poloxamer 407.

3.3.3 Splenocyte immune cell phenotypes.

We observed a similar distribution of T cells subsets amongst groups, including total T cells (CD3+), helper T cells (CD3+CD4+), cytotoxic/suppressor T cells (CD3+CD4-), activated helper T cells (CD4+CD80+), total B cells (CD19+) and proportion of helper T cells expressing proliferation and activation markers, including the IL-2 receptor (CD4+CD25+) (*Table 3.6*). Also, there was no significant difference in the proportion of macrophages (CD68+ or CD68+CD36+) expressing the toll-like receptor-4 (TLR-4; CD68+CD284+), and the adhesion molecule ICAM-1 (CD68+CD54+ or CD54+) (*Table 3.6*).

Table 3.6 Immune cell phenotypes from Ldlr$^{-/-}$ mice splenocytes fed with experimental diets for 12 wk

Cell Phenotype, % of total cells	CON (*n*=5)	CS (*n*=4)	PCS (*n*=5)	*p* Value
CD3+ (total T cell)	38.4 [32.6-43.4]	31.8 [27.3-46.8]	37.8 [34.4-42.8]	0.879
CD3+CD4+ (Helper T cells)	20.6 ± 2.7	20.2 ± 2.63	19.7 ± 1.6	0.846
CD4+CD25+ (Helper T cells expressing IL-2 receptor)	4.4 ± 0.7	5.1 ± 1.3	4.6 ± 1.0	0.567
CD4+CD80+ (Activated T cells)	5.6 ± 2.4	5.5 ± 1.8	9.1 ± 5.1	0.195
CD3+CD4- (Cytotoxic T cells)	12.1 ± 2.0	12.6 ± 5.3	13.8 ± 3.4	0.748
CD19+ (total B cell)	56.7 [54.4-62.0]	52.1 [34.2-67.3]	58.4 [57.4-59.3]	0.095
CD68+ (Macrophages)	8.5 [6.5-13.8]	13.0 [8.0-30.8]	14.0 [7.5-27.0]	0.070
CD68+CD36+ (Total Macrophages)	3.6 ± 0.8	4.0 ± 0.7	4.2 ± 0.6	0.298
CD68+CD284+ (Macrophages expressing the TLR-4)	5.0 ± 1.4	4.8 ± 2.0	4.9 ± 1.6	0.945
CD68+CD54+ (activated Macrophages)	6.0 ± 0.8	6.2 ± 1.2	6.4 ± 1.1	0.835
CD54+ (total ICAM-1)	40.4 ± 3.9	37.5 ± 3.2	39.6 ± 2.8	0.323

Values are presented as means ± SD or medians [95% CIs]. *p* value of the main effect of diet analyzed by one-way ANOVA followed by a Tukey's post-hoc test. Multiple comparisons between diet. Within experiment, labeled values in a row without a common superscript letter differ. Significance was set at $p<0.05$ in all analyses $p < 0.05$. CON, control; CS, choline-supplemented; ICAM-1, Intercellular Adhesion Molecule 1; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; PCS, phosphatidylcholine-supplemented; TLR-4, Toll-like receptor 4.

3.3.4 Peripheral blood immune cell phenotypes

We found that the distribution of activated T cells (CD4+CD80+), total B cells (CD19+) and proportion of T cells expressing proliferation and activation markers, including the IL-2 receptor (CD4+CD25+) were not different amongst dietary groups (*Table 3.7*). In addition, macrophages and their main activation markers (TLR-4 and ICAM-1); CD68+, CD68+CD36+, CD68+CD284+, CD68+CD54+, and CD54+) did not differ (*Table 3.7*).

Table 3.7 Immune cell phenotypes from Ldlr$^{-/-}$ mice peripheral blood fed with experimental diets for 12 wk

Cell Phenotype, % of total cells	CON (*n*=4)	CS (*n*=6)	PCS (*n*=6)	*p* Value
CD4+CD25+ (Helper T cells expressing IL-2 receptor)	0.5 ± 0.2	0.7 ± 0.3	0.8 ± 0.4	0.458
CD4+CD80+ (Activated T cells)	2.1 [1.5-3.3]	4.0 [1.8-8.0]	3.6 [2.8-4.8]	0.134
CD68+ (Macrophages)	8.7 ± 1.5	11.9 ± 3.4	9.8 ± 1.5	0.253
CD68+CD36 (Total Macrophages)	1.5 ± 0.4	2.2 ± 1.0	2.7 ± 0.8	0.107
CD68+CD284+ (Macrophages expressing the TLR-4)	0.5 ± 0.2	0.5 ± 0.3	0.4 ± 0.2	0.689
CD68+CD54+ (activated Macrophages)	4.5 ± 1.3	5.3 ± 1.8	5.1 ± 0.8	0.672
CD54+ (total ICAM-1)	21.2 ± 2.8	22.0 ± 2.8	21.7 ± 2.4	0.895

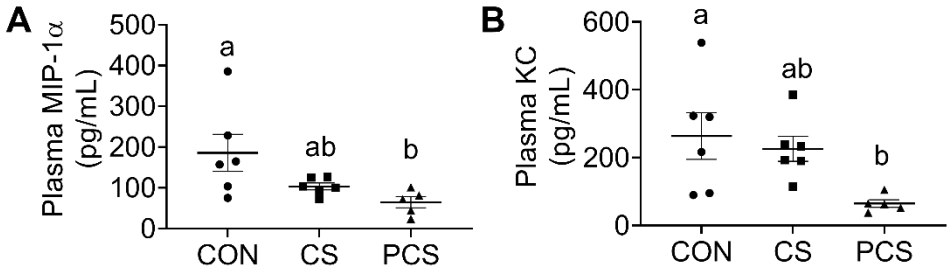
Values are presented as means ± SD or medians [95% CIs]. *p* value of the main effect of diet analyzed by one-way ANOVA followed by a Tukey's post-hoc test. Multiple comparisons between diet. Within experiment, labeled values in a row without a common superscript letter differ. Significance was set at $p<0.05$ in all analyses $p < 0.05$.

CON, control; CS, choline-supplemented; ICAM-1, Intercellular Adhesion Molecule 1; *Ldlr$^{-/-}$*, low-density lipoprotein receptor knockout; PCS, phosphatidylcholine-supplemented; TLR-4, Toll-like receptor 4.

3.3.5 Plasma chemokines and cytokines levels

We observed that plasma macrophage inflammatory protein-1α (MIP-1α), and keratinocyte chemoattractant (KC) levels were significantly lower in the PCS group compared to CON (*Figure 3.4 A, B*). These chemokines are known for having a pro-atherogenic effect by being secreted in later atherosclerotic burden stage as a consequence of the foam cell formation (Boisvert et al. 2006; Kennedy et al. 2012). However, there were no differences in cytokines typically related to atherosclerosis such as IFNγ, TNFα, IL-1β, IL-6, IL-2, and IL-10 (data not shown).

Figure 3.4 Plasma chemokines in Ldlr$^{-/-}$ mice



Plasma MIP-1α (A) and KC (B) from 20-22-wk-old male *Ldlr*$^{-/-}$ mice fed with CON, CS or PCS diet for 12 wk. Values are reported as means ± SEMs, n = 5-6. Groups without a common letter differ, $p<0.05$. CON, control; CS, choline-supplemented; KC, keratinocyte chemoattractant; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; MIP-1α, macrophage inflammatory protein-1α and PCS, phosphatidylcholine-supplemented.

3.3.6 Gene expression in whole aorta

Because we observed an increase in plasma HDL-C and lower levels of circulating pro-atherogenic chemokines in the PCS group, we investigated whether there are differences in mRNA abundance of genes involved in inflammation or cholesterol metabolism in the aortic root. We found that CS group had lower mRNA levels of IL-2, a T-cell proliferation and differentiation marker, compared to CON and PCS groups (*Table 3.8*). However, we did not observe any significant difference among dietary groups in the expression of genes related to macrophages, pro- and anti-inflammatory cytokines, cholesterol metabolism, adhesion molecules of smooth muscle cells. (*Table 3.8*).

Table 3.8 Aortic root mRNA levels from Ldlr$^{-/-}$ mice fed with experimental diets for 12 wk

Genes	CON (*n*=4)	CS (*n*=6)	PCS (*n*=6)	*p* Value
Macrophages				
Cd68	0.68 ± 0.13	0.81 ± 0.07	1.05 ± 0.44	0.138
F4/80	1.00 ± 0.64	1.06 ± 0.28	1.00 ± 0.63	0.977
Pro-inflammatory cytokines				
Tnfa	0.25 ± 0.08	0.32 ± 0.14	0.97 ± 0.81	0.086
Il-6	0.85 ± 0.54	1.57 ± 0.48	1.09 ± 0.47	0.109
Ifng	0.68 [0.21-1.28]	1.40 [0.21-1.28]	0.72[0.21-1.28]	0.065
Il-1b	1.00 ± 0.91	1.07 ± 0.32	1.16 ± 0.63	0.928
Anti-inflammatory cytokines				
Il-10	0.64 ± 0.05	0.70 ± 0.22	1.03 ± 0.58	0.279
Il-4	0.77 ± 0.23	0.81 ± 0.34	0.81 ± 0.40	0.980
Cell proliferation				
Il-2	1.12 ± 0.18^{a}	0.26 ± 0.13^{b}	0.90 ± 0.23^{a}	<0.0001
Cholesterol uptake				
Cd36	0.75 ± 0.34	0.72 ± 0.19	1.22 ± 0.82	0.273
Msr1	1.00 ± 0.54	1.06 ± 0.17	1.05 ± 0.79	0.98
Scarb1	1.00 ± 0.35	1.04 ± 0.11	0.91 ± 0.26	0.71
Cholesterol efflux				
Abcg1	1.00 ± 0.34	1.08 ± 0.14	0.97 ± 0.51	0.900
Abca1	0.82 ± 0.16	0.91 ± 0.06	0.94 ± 0.34	0.712
Cholesterol metabolism				
Olr1	1.00 ± 0.37	1.46 ± 0.55	1.20 ± 0.82	0.465
Acat1	1.00 ± 0.34	1.10 ± 0.17	0.80 ± 0.14	0.194
Acat2	1.00 ± 0.48	1.15 ± 0.29	1.23 ± 0.69	0.748
Adhesion molecules				
Vcam-1	0.34 [0.27-0.47]	0.45 [0.34-0.53	0.74 [0.003-1.93]	0.191
Icam-1	0.76 ± 0.15	1.00 ± 0.18	1.21 ± 0.59	0.202
Smooth muscle cells				
Cd45	0.09 ± 0.05	0.23 ± 0.04	1.26 ± 1.81	0.052

Values are presented as means ± SD or medians [95% CIs]. *p* value of the main effect of diet analyzed by one-way ANOVA followed by a Tukey's post-hoc test. Multiple comparisons between diet. Within experiment, labeled values in a row without a common superscript letter differ. Significance was set at $p<0.05$ in all analyses $p < 0.05$.

CON, control; CS, choline-supplemented; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; PCS, phosphatidylcholine-supplemented.

3.4 Discussion

The current study demonstrates for first time that dietary PC supplementation (3x typical amount of choline in rodent diets) significantly decreased atherosclerosis development in *Ldlr*$^{-/-}$ mice. Our data is consistent with early work from Samochowiec et al. who showed that PC administration (orally or directly into the stomach) reduced atherosclerosis in miniature pigs and white rats by reducing serum levels of total lipids, TG, cholesterol, and APOB (Samochowiec, Kadłubowska, Różewicka, et al. 1976; Samochowiec, Kadłubowska, and Różwicka 1976). However, it should be noted that these studies did not control for the choline or fatty acid content of the diet. In the present study, plasma VLDL-cholesterol was reduced and plasma TMAO levels were elevated in PCS group. CS diet did not affect plasma TMAO levels or atherosclerosis development. In our previous study, we reported that feeding two atherogenic mouse (*Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$) models with a choline-supplemented diet (10x normal) did not alter the size of atherosclerotic lesions, despite increasing plasma TMAO levels (Aldana-Hernández et al. 2020). Dietary choline and PC are metabolized and absorbed by enterocytes through different mechanisms. Approximately, ~80% of dietary choline is absorbed by specific transporters and by passive diffusion throughout the small intestine (Sheard and Zeisel 1986; Zeisel et al. 1983), while PC is hydrolyzed to lysoPC in the intestinal lumen by pancreatic phospholipase A2, then lysoPC is absorbed by the enterocyte (Arnesjö et al. 1969). The differences in plasma TMAO levels between CS and PCS diet might indicate that free choline is more rapidly absorbed and thus not metabolized by the bacteria in the lumen of the intestine, whereas slower absorption of lyso-PC might allow it to be broken down to free choline in the lumen by the bacterial enzyme phospholipase D1 (Chittim et al. 2019). The resulting free choline from PC would then become available for the bacterial TMA-lyases leading to an increase in plasma TMA levels (Gao, Jiang, et al. 2016). Nevertheless, our data confirm several recent

animal studies that increased plasma TMAO concentrations did not directly increase atherosclerotic lesion size in atherogenic mouse models (Aldana-Hernández et al. 2020; Huc et al. 2018; Lindskog Jonsson et al. 2018). In a recent randomized, controlled, double-blinded, crossover dietary intervention, 37 men received a single meal with *i)* no choline control (42 mg of choline), *ii)* choline bitartrate (600 mg of choline), and *iii)* soy PC (600 mg of choline) supplemented tomato soup on 3 different visits (Cho et al. 2020). Plasma postprandial and urine TMAO concentrations were elevated after eating the free choline meal compared to the no choline control and the PC supplemented meals (Cho et al. 2020). In humans, long-term consumption of up to 3 eggs daily for 4 weeks did not alter plasma TMAO levels (Lemos et al. 2018b; Missimer et al. 2017). However, the long-term effects of dietary choline versus PC supplementation on TMAO levels need further examination in humans. In our study, plasma choline and betaine levels remained similar in the PCS group compared to CON and CS groups. Choline metabolism occurs mainly in the liver and subproducts such betaine and dimethylglycine are important for one-carbon metabolism (Ueland 2011), future studies are needed to understand the direct interaction on dietary PC supplementation on the one-carbon metabolism.

Consistent with previous animal studies, we observed an increase in body weight in mice fed a PCS diet (Dellschaft et al. 2018; Lewis et al. 2017; Richard, Lewis, et al. 2017). More specifically, BAT weight was higher in the PCS group. Worthmann et al. reported that *Ldlr*$^{-/-}$ mice fed a high-fat, high-cholesterol, and high-sucrose-diet for 12 weeks, then switched to a chow diet supplemented with a BAT activator (β3-adrenoceptor agonist) resulted in an improvement in hyperlipidemia, but not atherosclerosis development (Worthmann et al. 2019). In our study, gonadal fad pads trended higher in the PCS group, which might suggest that the higher storage fat in periphery adipose tissue lowered atherosclerosis development.

Mice in the PCS group had smaller atherosclerotic lesions even though plasma cholesterol levels remained similar to the CON group. Interestingly, the PCS group had higher HDL-C and lower VLDL-C compared to CON and CS groups. Other studies have shown that dietary PC has a role in HDL-C regulation (Jimenez et al. 1990; Wilson, Meservey, and Nicolosi 1998). Similar to our study, Jimenez et al showed that adult rats fed for 30 days with hypercholesterolemic diet (1% cholesterol+0.5% cholic acid w/w) with soy lecithin (2.5% w/w) had a reduction in plasma VLDL-C and an increase in plasma HDL-C (Jimenez et al. 1990). These effects were associated to an increase of lecithin-cholesterol acyltransferase (LCAT) activity in plasma (Jimenez et al. 1990). LCAT is responsible for esterifying the free cholesterol in plasma HDL (Shiomi, Koike, and Ishi 2012). However, we did not observe any changes in hepatic *Lcat* expression. APOA1 is the major apolipoprotein present in HDL particle and is a key player in the reverse cholesterol transport (Davidson and Thompson 2007). Unexpectedly, we observed that APOA1 was lower in PCS group compared to CON group. APOE is an apolipoprotein present on HDL particles and is often associated with increased particle size and cholesterol content; however, plasma APOE amount did not change in the PCS group, and the FPLC profile did not reveal an obvious increase in particle size. Thus, it seems that dietary PC supplementation modified the composition of HDL particles, with increased cholesterol but lower APOA1 content. It is possible that PC supplementation could change the phospholipid composition of HDL particles, making them better acceptors for macrophage-derived cholesterol. This would increase the cholesterol content of mature HDL particles and contribute to a reduction in atherosclerotic plaque formation. Another aspect that needs to be contemplated is that both APOA1 and APOB48 are present in chylomicrons (Feingold and Grunfeld 2000). Recent studies *in vivo* and *in vitro*, have shown that PC infusion or

dietary supplementation impaired cholesterol absorption in the intestine (Lee et al. 2019; Yang et al. 2018). However, this is speculative and would be subject to future studies.

In this study, we observed that PCS diet reduced plasma VLDL-C without changing plasma VLDL-TG fraction. It is well known that phospholipids are important for the assembly and secretion of lipoprotein particles (Gibbons et al. 2004; Noga and Vance 2003b; Rava et al. 2006; Shelness and Ledford 2005; van der Veen, Kennelly, et al. 2017). In both the liver-specific CTα and PEMT knockout mice, impaired VLDL secretion was linked to reduced hepatic PC/PE ratio (Jacobs et al. 2004; Zhao et al. 2009). Furthermore, secreted VLDL that have a reduced PC/PE ratio are cleared faster from the plasma (Zhao et al. 2009). It is likely that dietary supplementation of PC changed the amount of PC on chylomicron or VLDL particles. It is possible that the PCS diet increases clearance of APOB-containing lipoproteins; however, this was not directly determined in our experiments.

The development of atherosclerosis is a multifactorial process and does not only depend on lipoprotein metabolism. Immune cells and inflammatory mechanisms are strongly involved in the atherosclerosis process by the recruitment of monocytes/macrophages responsible for the clearance of atherosclerotic plaque (Gisterå and Hansson 2017; Hansson 2001). Several studies in rats have shown that dietary PC supplementation in dams improves immune development and function in their offspring (Dellschaft et al. 2015; Lewis et al. 2017; Lewis, Richard, et al. 2015; Richard, Lewis, et al. 2017). We did not observe any effect on immune cell phenotypes, which suggests that the effect of PC on immune function might have more impact during the prenatal stage of development than during adulthood. Nevertheless, we observed lower plasma levels of two pro-atherogenic chemokines: MIP-1α, and KC in the PCS mice. Kennedy et al. investigated the role of MIP-1α in atherosclerosis. *Ldlr*$^{-/-}$ mice were transplanted with bone marrow from mice

lacking MIP-1α and were fed a Western diet. Transplanted mice had lower plasma cholesterol and TG levels, leading to reduced atherosclerotic lesion size (Kennedy et al. 2012). KC is one of the two chemo-attractants responsible for recruiting neutrophils that bind to chemokine receptors in response to atherosclerosis (Huo et al. 2001). Consistent with this, chimeric *KC/GROα*$^{-/-}$*/Ldlr*$^{-/-}$ mice had lower atherosclerotic lesion size (Boisvert et al. 2006).

In summary, this study demonstrates that dietary PC supplementation decreased atherosclerosis development, despite an increase in plasma TMAO concentration in an atherogenic mouse model. Changes in lipoprotein metabolism might partly explain the reduction in atherosclerotic plaque; however, the mechanism behind this observation is unclear. Our results highlight the importance of considering the different forms of choline in the diet for the improvement of atherosclerosis development.

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Chapter 4: Examining the role of different dietary sources of choline on postprandial choline and TMAO metabolism in humans: a pilot randomized controlled crossover trial

4.1 Introduction

In 1998, choline was established as essential nutrient required for critical processes including lipoprotein synthesis, methyl group donation, neurotransmitter synthesis and cell membrane formation (Institute of Medicine 1998; Zeisel et al. 1991; Zeisel and da Costa 2009). A choline deficient diet result in fatty liver disease, and negatively affect offspring brain and immune system development (Buchman et al. 1995; Dellschaft et al. 2018; Institute of Medicine 1998; Lewis, Richard, et al. 2015; Richard, Lewis, et al. 2017). Choline is found in a variety of foods as different forms; phosphatidylcholine (PC) and free-choline (FC) being the two most common in the diet of North American population (Yonemori et al. 2013). The recommended adequate intake (AI) is 550 and 425 mg/d for men and women, respectively (Institute of Medicine 1998). Studies have shown that the intake of choline is below the AI in various populations worldwide (Anon 2016; Elizabeth N. Pearce 2016; Gao, Wang, et al. 2016; U.S. Department of Agriculture 2020; Wallace et al. 2018; Wallace and Fulgoni 2017). In order to meet the AI it has been recommended to increase the consumption of choline rich foods including eggs, dairy and meat (Patterson et al. 2008b). However, it has been suggested that consumption of choline rich foods (specifically PC from animal source) may increase cardiovascular disease (CVD) risk (Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; Lever et al. 2014; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; Tang et al. 2015; Tang et al. 2015b; Wang et al. 2011, 2014; Zhu et al. 2016). In theory, excess dietary choline reaches the large intestine and is metabolized by gut microorganisms to trimethylamine (TMA) and then is further oxidized in the liver to form trimethylamine N-oxide (TMAO) (de la Huerga and Popper 1951; Lang et al. 1998; Romano et al. 2015; Wang et al. 2011; Zeisel 1981; Zhang et al. 1999). Epidemiological studies suggest that plasma TMAO is a biomarker for

atherosclerosis (Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; Lever et al. 2014; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; Wilson Tang et al. 2015; Tang et al. 2015b; Wang et al. 2011, 2014; Zhu et al. 2016). Recently, we reported that dietary choline supplementation (10X recommendation) increased plasma TMAO levels but it did not induce atherosclerosis development in two mouse models (Aldana-Hernández et al. 2020; Lindskog Jonsson et al. 2018). To date, cross-sectional and prospective studies in humans have not observed a significant association between dietary choline intake and CVD risk (Meyer and Shea 2017). Eggs are a major source of PC and studies have shown that long term consumption up to 3 eggs daily for 4 weeks did not alter plasma TMAO levels (DiMarco et al. 2017; Lemos et al. 2018; Missimer et al. 2018; Zhang et al. 1999). Interestingly, foods rich in choline, carnitine or TMAO content (such eggs, fish) have reported an increase postprandial plasma TMAO levels (Cho et al. 2017, 2020; Hagen et al. 2020; Miller et al. 2014). However, the meals provided were not standardized for energy, fat or fibre content. The aim of this study was to examine the effect of standardized breakfasts containing different dietary forms and sources of choline on postprandial choline and TMAO metabolism in healthy men. We hypothesized that the source and amount of dietary choline would have no effect on TMAO production or status following a meal but will alter choline concentration in plasma.

4.2 Material and methods

4.2.1 Subjects and study design

This study was a randomized controlled crossover design with four arms composed of breakfasts differing in the form of dietary choline. Eight healthy men age 18-59 years with a with a body mass index (BMI) 18.5–25.0 kg/m^2 were recruited for the study. Being a smoker, having history

of chronic disease, fearing of needles, not eating breakfast, having food allergies, disliking study foods, and being vegetarian and vegan were cause for exclusion. A sample size of n = 8 was determined from a power analysis of a within-subject design to detect a 50% difference of plasma TMAO concentration at $\alpha < 0.05$ and $\beta = 0.8$. Women were excluded due to FMO activity is increased by estrogen (Bennett et al. 2013). Posters around the University of Alberta campus were posted. Possible participants were contacted via phone call to administrate a screening questionnaire for inclusion criteria and to set a pre-study visit at the Human Nutrition Research Unit (HNRU). Exclusion criteria were men of age >60 years, BMI >25 kg/m2, women, vegetarians, smokers, and individuals with gastrointestinal diseases or complaints, chronic illnesses or other metabolic diseases. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects were approved by the University of Alberta Health Research Ethics Biomedical Panel (protocol #Pro00068657). A written informed consent was obtained from all subjects prior the intervention.

During the pre-study visit, participants were informed about all procedures for the study and subsequent four visits. Participants were asked to provide information about their age and health condition. Body weight and height were measured within the research unit. Participants were instructed to avoid consumption of grapefruit and indole-containing vegetables (including broccoli, cauliflower, kale, brussels sprouts, cabbage and bok choy) in the day prior to the study visit, as these foods have been shown to alter TMAO metabolism by decreasing the flavin containing monooxygenase enzyme activity.

For the first study visit, participants were asked to fast overnight for 12 h and come to the HNRU. Randomly participants were assigned to each of the four study breakfasts. Participants consumed

each of the four study breakfasts by returning to the HNRU for subsequent visits with at least one-week of wash out period in between.

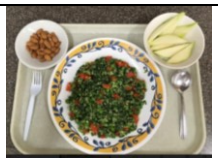
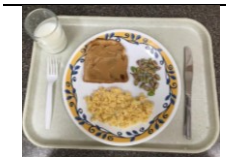
At the beginning (time point 0, T0) of each study visit an intravenous (IV) catheter was inserted and blood was collected in 3mL EDTA vacuum tubes EDTA. Then participants consumed the assigned study breakfast within a 15 min. To measure postprandial choline-metabolites response, blood was collected after finishing the meal by using an IV at 0.5, 1, 2, 4, 6, and 8 h postprandial, while participants remained at the HNRU. At the 4 h timepoint collection, participants were given a low choline snack. After each blood collection, tubes were placed on ice until centrifuged at 3500 rpm for 10 min to separate plasma. Plasma samples were aliquoted into separate tubes and stored at -80°C.

4.2.2 Breakfast description

To have standardized meals and to control the effect of gastric emptying and digestion speed the study breakfasts were similar in calories, fat, protein, and fibre (*Table 4.1*). The Standard Operating Procedures for the preparation of each meal are described in Appendix 1, 2, 3, and 4. The University of Alberta Database for the Choline Content of Common Foods (Alberta Database) (described in detailed in Appendix 6) was used to determine the choline and betaine content of the four breakfasts (Lewis 2016). Three study breakfasts contained approximately 363 mg of total choline from different sources or form: high PC meal (HPCM) with~ 91% of choline as PC, high PC from supplement (HPCS) with ~97% as PC (soy lecithin granules code 005648 from GNC), and high FC (HFC) with ~ 89% as FC (trophic choline bitartrate supplement) (*Table 4.1*). The HPCM breakfast provided the majority of choline as PC from food and consisted of fried whole eggs, toast, bacon, blackberries, raspberries, and fibre supplement (2/3 microcrystalline cellulose (Microcel) Identification Code: MC-12 from BLANVER and 1/3 resistant starch (Fibersym RW)

CAS No. 977043-58-5 from MGP Ingredients, Inc). The HPCS breakfast consisted of an egg white omelet with spinach, red pepper and green pea, almonds, pear, soy lecithin granules (code 005648 from GNC), in which the choline was mainly as PC from plant sources. The HFC breakfast was a cheddar cheese egg white omelet, toast with peanut butter, skim milk, a clementine, pistachios, choline bitartrate supplement (Trophic choline bitartrate) and fibre supplement. A low choline (LCM) breakfast was designed as a control containing approximately 15 mg of total choline and consisted of a blueberry pancake with egg whites, fibre supplement, and butter (*Table 4.1*). On each study visit participants received a low choline snack, yogurt, and apple after 4 h of eating the breakfast (Standard Operating Procedures for the preparation of the low choline snack is describe in Appendix 5).

Table 4.1Breakfast description and nutrient components

Breakfast	LCM	HPCM	HPCS	HFC
Description	 Blueberry pancakes Egg whites Butter Fibre supplement[1]	 Whole eggs Bacon Whole wheat bread Butter Blackberries Raspberries Fibre supplement[1]	 Spinach, red pepper, and green pea egg white omelette Olive oil Almonds Pear Soy lecithin granules[2]	 Cheese egg white omelette Whole wheat bread Peanut butter Skim milk Clemetine Pistachios Choline bitartrate supplement[3] Fibre supplement[1]
Total choline, mg	15	363 (91% as PC)	364 (97% as PC)	363 (89% as FC)
Choline AI %	2.7	66	66	66
Total energy, kcal	461	449	453	459
Betaine, mg	9.3	30	33	30
Total fat, g (% kcal)	24.6 (48)	25.1 (50)	25.7 (51)	24.6 (48)
Total protein, g (% kcal)	24.6 (21)	25.1 (22)	24.7 (22)	25.3 (22)
Total dietary fibre, g	11.3	11.4	11.4	11.2

[1] Fibre supplement content was: 2/3 microcrystalline cellulose (Microcel) Identification Code: MC-12 from BLANVER and 1/3 resistant starch (Fibersym RW) CAS No. 977043-58-5 from MGP Ingredients, Inc.
[2] Soy lecithin granules code 005648 from GNC.
[3] Trophic choline bitartrate supplement
Adequate intake, AI; free-choline, FC; Low choline, LCM; high PC meal, HPCM; high free-choline, HFC; high PC from supplement, HPCS

4.2.3 Dietary intake

During each study visit participants completed a 24-h dietary intake recall, in which in detail everything they ate and drank in a 24-h period prior to the study visit were recorded. These 24 h dietary intake recalls were conducted in face-to-face interviews by trained nutritionist using a 'multiple-pass method'(Conway et al. 2003). Food models were used to estimate portion sizes and probes including details regarding cooking method and food brand names were used. Participants received a copy of their 24-hour dietary recall, and asked to maintain their normal routine, including exercise, and eat similarly to this day for the remaining study visits. Data collected during the 24 h dietary intake recall interviews were entered into Food Processor Standard Query Language (ESHA Research) to estimate macro- nutrient intake.

Dietary choline intake was determined by using the University of Alberta Database for the Choline Content of Common Foods (Alberta Database) (Standard Operating Procedures for use of the University of Alberta Database for the Choline Content of Common Foods (Alberta Database) is described in detailed in Appendix 6) (Lewis 2016). Briefly, the database contains information of total choline content in food, the five most common dietary choline forms (FC, PC, glycerophosphocholine, phosphocholine, and betaine). The Alberta Database includes choline values for food items from the USDA Database for the Choline Content of Common Foods Release 2 (634 foods) and USDA National Nutrient Database for Standard Reference (Zeisel, Mar et al. 2003, Patterson, Williams et al. 2008). For missing items, 26 new foods were added to the database through analysis by hydrophilic interaction liquid chromatography–tandem mass spectrometry (Lewis, Zhao, et al. 2015; Xiong et al. 2012; Zhao et al. 2011). Intakes of macro and micronutrients were adjusted to 1,000 Kcal.

4.2.4 Plasma metabolite analysis

Plasma total cholesterol and triacylglycerol (TG) levels were measured by commercially available kits from Wako (Catalog No. 999-02601) and SEKISUI Diagnostics (Catalog No. 236-60), respectively. Plasma choline, betaine, TMA and TMAO were quantified by hydrophilic interaction liquid chromatography–tandem mass spectrometry as previously described (Mi et al. 2017; Xiong et al. 2012; Zhao et al. 2011) .

4.2.5 Statistical analysis

All statistical analyses were performed using the Graph Pad Prism 8. Data was tested for normal distribution by Shapiro-Wilk normality test. Data that were not normally distributed, were Log-transformed for the analysis. A mixed-paired model followed by a Tuckey's post hoc was used to test the effect of breakfast and postprandial time on each outcome. The postprandial effect of amount, type and source of PC was analyzed with paired Student's T-test. Values at baseline were set as 100% to compare the change with each timepoint. Low- and High-TMAO responders were establish with median (1.15 μM). The baseline choline-metabolites and dietary intakes were analyzed with unpaired Student's T-test. For all measures, $p < 0.05$ was accepted as statistically significant. Results are presented as means ± standard errors of means or medians [95% CIs].

4.3 Results

4.3.1 Participant characteristics

Eight healthy men participated in this study. Participant characteristics at baseline are shown in *Table 4.2*. Participants were grouped as Low- and High-TMAO baseline. An analysis of the baseline plasma choline-metabolites in Low- and High-TMAO baseline is presented in *Table 4.3*. High-TMAO baseline had higher plasma TMAO levels, and plasma betaine compared to Low-

TMAO baseline (*Table 4.3*). However, plasma TMA and choline levels were similar between groups (*Table 4.3*).

4.3.2 Dietary intake

The average dietary intakes prior to 24-h visit remained consistent for each participant across the study. There were no differences in energy intake, carbohydrates, protein, and fat (% kcal), and fibre (g/d). Betaine and choline-moieties intake were similar among the participants prior to each visit (*Table 4.4*). Choline AI of 550 mg/d was reached by 1, 2, 1, and 3 participants the day before LCM, HPCM, HPCS, and HFC breakfast intervention, respectively (*Table 4.4*). An analysis of the dietary intake in Low- and High-TMAO baseline is presented in *Table 4.5*. High-TMAO baseline showed significantly higher trans-fatty acids intake compared to Low-TMAO baseline (*Table 4.5*). We did not find differences in other nutrients intakes (*Table 4.5*).

4.3.3 Postprandial effect on plasma TMA and TMAO levels

The breakfasts provided to the participants did not influence postprandial TMA levels (*Figure 4.1 A, B*). However, 2 and 4 h after consuming HFC there was increased ~2X of plasma TMA compared to HPC breakfast (*Figure 4.1 E, F*).

The postprandial response on plasma TMAO did not change significantly with the breakfast provided (*Figure 4.2 A, B*). However, plasma TMAO levels were significantly higher after 0.5 h of having the HFC breakfast compared to the HPCS breakfast (*Figure 4.2 A*). After 0.5 h of consuming the HPCS breakfast plasma TMAO levels were significantly higher compared to HPCM breakfast (*Figure 4.2 G*).

4.3.4 Postprandial effect on plasma choline and betaine levels

Postprandial choline response was not different among the breakfasts (*Figure 4.3 A, B*). However, plasma choline was higher after 4 h consuming the HPCM breakfast compared to LCM and HPCS breakfasts (*Figure 4.3 A, G*).

The breakfasts provided with high choline content were also high in betaine (*Table 4.1*). The postprandial betaine levels were influenced by the breakfasts (*Figure 4.4*). Postprandial plasma betaine response was increased after consuming the HC breakfasts (high in betaine content) compared to LCM breakfast *(Figure 4.4 A, C and D*). The type of choline from the HC breakfasts did not change the postprandial betaine levels (*Figure 4.4 E*). Even though analyzing the source of choline, the HPCM breakfast showed higher betaine postprandial effect than the HPCS breakfast (*Figure 4.4 G*).

4.3.5 Postprandial plasma TMAO response in Low and High TMAO baseline groups

Next, we analyzed the postprandial TMAO response on TMAO Low and High TMAO baseline groups. In the Low TMAO baseline group plasma TMAO levels were significantly higher after 0.5 h of consuming the HFC compared to the LCM meal (*Figure 4.5 A* and *C*). After 2 h of having the HPCM plasma TMAO was higher than after consuming the HFC meals (*Figure 4.5 A* and *C*). In the Low TMAO group plasma TMAO level were higher with the HFC compared to HPCS breakfast after 1 h (*Figure 4.5 C, G,* and *I*). Meanwhile, the postprandial response on plasma TMAO in the High TMAO baseline group remain similar among the breakfasts (*Figure 4.5 B, C*).

Table 4.2 Characteristics at baseline of participants enrolled in a randomized controlled crossover trial examining the role of different dietary sources of choline on postprandial choline and TMAO metabolism

Age (years)[1]	33 ± 9.3
Weight (Kg)[1]	73 ± 12
BMI (Kg/m^2)[1]	24 ± 3.0
Plasma cholesterol (mM)[2]	5.5 ± 1.5
Plasma TG (mM)[2]	1.2 ± 0.7
Plasma choline (mM)[2]	4.0 ± 2.4
Plasma betaine (mM)[2]	26 ± 8.0
Plasma TMA (μM)[2]	0.5 ± 0.6
Plasma TMAO (μM)[2]	2.7 ± 3.8
Plasma TMA/TMAO[2]	0.8 ± 2.1

Values are presented as means ± SD, n= 8.
[1]Data collected at the pre-visit.
[2]Data collected at the first visit.
Trimethylamine, TMA; trimethylamine N-oxide, TMAO.

Table 4.3 Plasma choline-metabolites at baseline in Low- and High-TMAO baseline

Parameter	Low TMAO baseline (n=4)	High TMAO baseline (n=4)	p value
Plasma TMAO, μM	0.89 [0.66-1.24]	1.74 [0.98-6.18]	0.0005***
Plasma TMA, μM	0.11 [0.11-0.79]	0.12 [0.13-0.81]	0.6622
Plasma choline, μM	4.7 [3.2-5.4]	3.2 [2.1-5.0]	0.1825
Plasma betaine, μM	32 ± 1.5	24 ± 2	0.0024**

[1] Participants were grouped in Low- and High-TMAO baseline.
Values were polled and analyzed at baseline of each visit day.
Values are presented as means ± SEM or medians [95% CIs], n= 4.
Low- and High-TMAO responders were analyzed by unpaired Student's T-test.
** $p<0.01$
*** $p<0.001$
Trimethylamine, TMA; trimethylamine N-oxide, TMAO.

Table 4.4 Dietary characteristics collected using 24 h dietary intake recall data on each visit[1]

Nutrient	LCM	HPCM	HPCS	HFC	*p* value
Energy intake, kcal/d	2844 ± 397	2856 ± 445	2667 ± 289	2749 ± 430	0.9359
Carbohydrate, g/d (% cal) [2]	107 ± 16 (43 ± 6)	132 ± 9 (53 ± 4)	126 ± 120 (51 ± 5)	125 ± 9 (50 ± 4)	0.1953 (0.1953)
Fibre, g/d [2]	2.6 [0.0-12.0]	2.6 [0.0-28.7]	2.5 [0.0-19.4]	3.6 [0.0-11.8]	0.1187
Protein, g/d (% cal) [2]	40 ± 5 (16 ± 2)	39 ± 5 (16 ± 2)	43 ± 4 (17 ± 2)	41 ± 6 (16 ± 2)	0.8223 (0.8223)
Fat, g/d (% cal) [2]	46 ± 5 (41 ± 4)	35 ± 3 (32 ± 3)	36 ± 5 (32 ± 5)	37 ± 4 (33 ± 4)	0.2482 (0.2482)
Saturated fatty acids, g/d [2]	14 ± 2	13 ± 1	13 ± 2	13 ± 2	0.8693
Monounsaturated fatty acids, g/d [2]	15 ± 3	9 ± 2	10 ± 2	11 ± 2	0.0661
Polyunsaturated fatty acids, g/d [2]	7.7 ± 1.2 [a]	4.4 ± 0.7 [b]	5.1 ± 1.4 [b]	3.8 ± 0.8 [b]	0.0388
Trans-fatty acids, g/d [2]	0.40 [0.10-2.39]	0.40 [0.10-0.96]	0.29 [0.04-2.00]	0.18 [0.03-0.73]	0.1669
Cholesterol, mg/d [2]	128 [35-215]	128 [35-243]	83 [23-190]	66 [3-426]	0.3835
Betaine, mg/d [2]	40 [2-216]	40 [2-190]	59 [10-146]	69 [18-391]	0.2772
Choline-containing moieties, mg choline/d [2]					
FC	27 [7-71]	27 [7-79]	107 [53-398]	40 [15-174]	0.4277
Glycerophosphocholine	19 [5-33]	19 [5-114]	19 [11-61]	29 [7-97]	0.5619
Phosphocholine	4.2 [0.6-10.8]	4.2 [0.6-20.5]	5.5 [2.1-7.4]	5.0 [0.4-8.4]	0.8996
PC	56 [9-136]	56 [9-256]	51 [28-92]	63 [12-317]	0.5065
Sphingomyelin	7.5 [0.4-18.8]	7.5 [0.4-24.5]	4.9 [0.0-19.4]	4.0 [1.3-33.0]	0.7816
Total choline [2]	107 [53-268]	107 [53-398]	132 [68-175]	156 [73-535]	0.3964
Meeting choline AI (550 mg/d)					
n (%)	1 (13)	2 (25)	1 (13)	3 (38)	NA

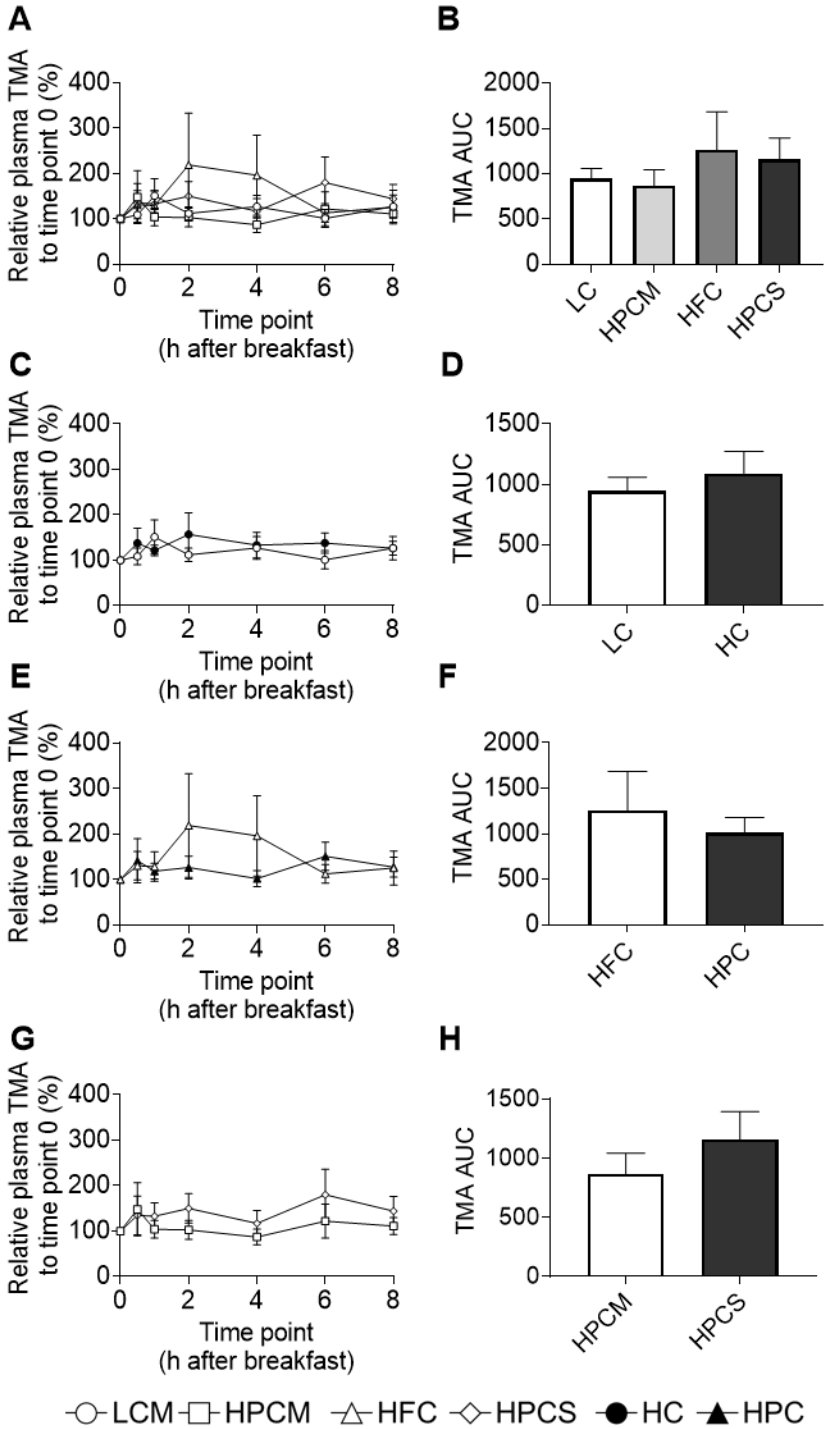
[1] All values were polled and analyzed for each day for each participant.
[2] Intakes of macro and micronutrients were adjusted to 1,000 Kcal 24 h recall before the breakfast intervention.
Values were polled and analyzed for each visit day.
Values are presented as means ± SEM or medians [95% CIs], n= 8.
Data were analyzed by a mixed-paired model followed by a Tuckey's post hoc.
Labeled values in a row without a common superscript letter differ.
Significance was set at $p<0.05$ in all analyses.
AI, Adequate intake; free-choline, FC; Low choline, LCM; high PC meal, HPCM; high free-choline, HFC; high PC from supplement, HPCS; phosphatidylcholine, PC.

Table 4.5 Dietary characteristics collected using 24 h dietary intake recall data on Low- and High-TMAO baseline

Nutrient	Low TMAO responders (n=4)	High TMAO responders (n=4)	p value
Energy intake, kcal/d	2879 ± 520	2679 ± 375	0.7647
Carbohydrate, g/d (% cal) [2]	132 ± 9 (53 ± 4)	113 ± 18 (45 ± 7)	0.3727 (0.3727)
Fibre, g/d [2]	6 [0.1-13]	2 [-5-13]	0.2469
Protein, g/d (% cal) [2]	37 ± 5 (18 ± 2)	45 ± 6 (15 ± 2)	0.2997 (0.2997)
Fat, g/d (% cal) [2]	37 ± 2 (36 ± 5)	40 ± 6 (33 ± 2)	0.6576 (0.6576)
Saturated fatty acids, g/d [2]	14 [9-17]	14 [7-19]	0.8784
Monounsaturated fatty acids, g/d [2]	11 ± 2	12 ± 4	0.8537
Polyunsaturated fatty acids, g/d [2]	5 ± 0.9	5 ± 1.3	0.9054
Trans-fatty acids, g/d [2]	0.3 ± 0.05	0.7 ± 0.13	0.0336*
Cholesterol, mg/d [2]	79 ± 17	137 ± 44	0.2542
Betaine, mg/d [2]	99 ± 10	81 ± 27	0.5702
Choline-containing moieties, mg choline/d [2]			
FC	41 ± 3	39 ± 10	0.8423
Glycerophosphocholine	29 ± 3	27 ± 6	0.8025
Phosphocholine	6 ± 1.0	5 ± 0.4	0.5600
PC	62 ± 22	100 ± 30	0.3343
Sphingomyelin	7 ± 2	12 ± 4	0.2993
Total choline [2]	145 ± 28	184 ± 35	0.4141
Meeting choline AI (% of 550 mg/d)			
n (%)	1 (25)	1 (25)	

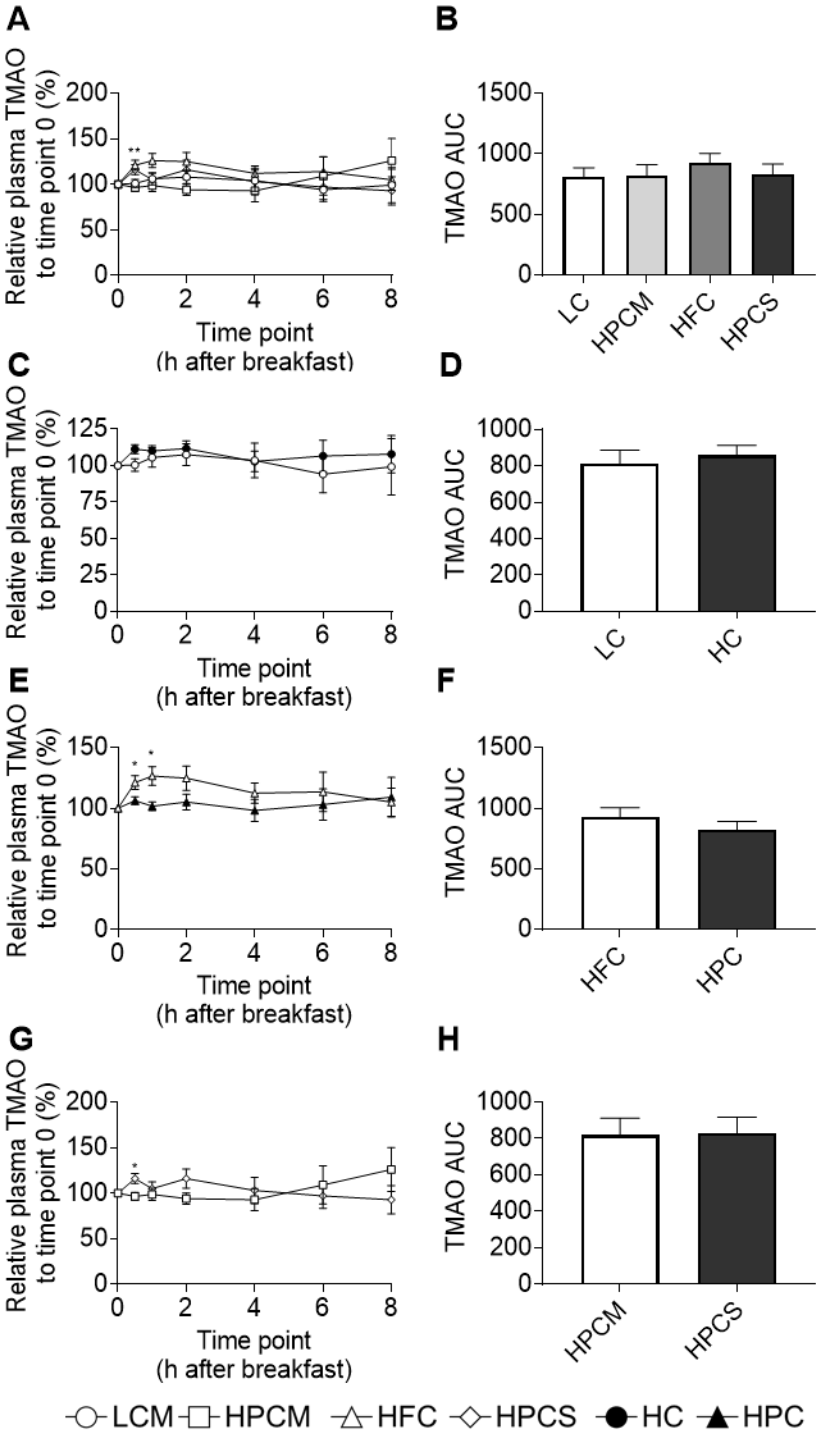
[1] Participants were grouped in Low- and High-TMAO responders.
[2] Intakes of macro and micronutrients were adjusted to 1,000 Kcal 24 h recall before the breakfast intervention.
Values were polled and analyzed for each visit day.
Values are presented as means ± SEM or medians [95% CIs], n= 4.
Low- and High-TMAO responders were analyzed by unpaired Student's T-test.
Significance was set at *p<0.05 in all analyses.
AI, Adequate intake; free-choline, FC; phosphatidylcholine, PC; Trimethylamine, TMA; trimethylamine N-oxide, TMAO.

Figure 4.1 Postprandial TMA response



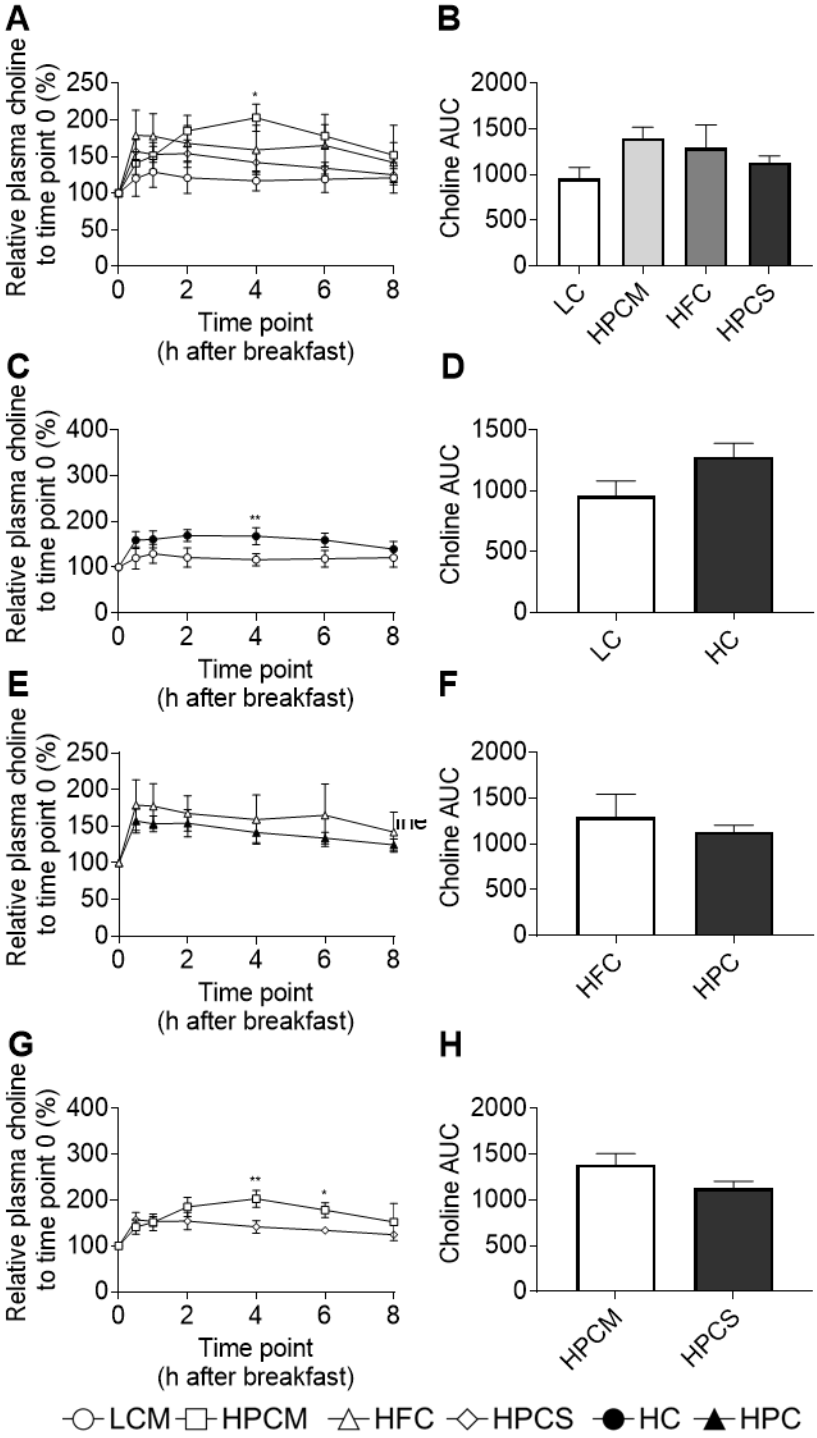
Postprandial plasma TMA from type of breakfast and AUC, n=8 (A, B), amount of choline grouped breakfasts and AUC, n=8/24 (C, D), type of choline grouped breakfasts and AUC, n=8/16 (E, F), and source of PC breakfasts and AUC, n=8 (G, H). Data are presented as mean ± SEM, n = 8-24. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Low choline, LCM; high PC meal, HPCM; high free-choline, HFC; high PC from supplement, HPCS; high choline, HC; high PC, HPC; trimethylamine, TMA.

Figure 4.2 Postprandial plasma TMAO response



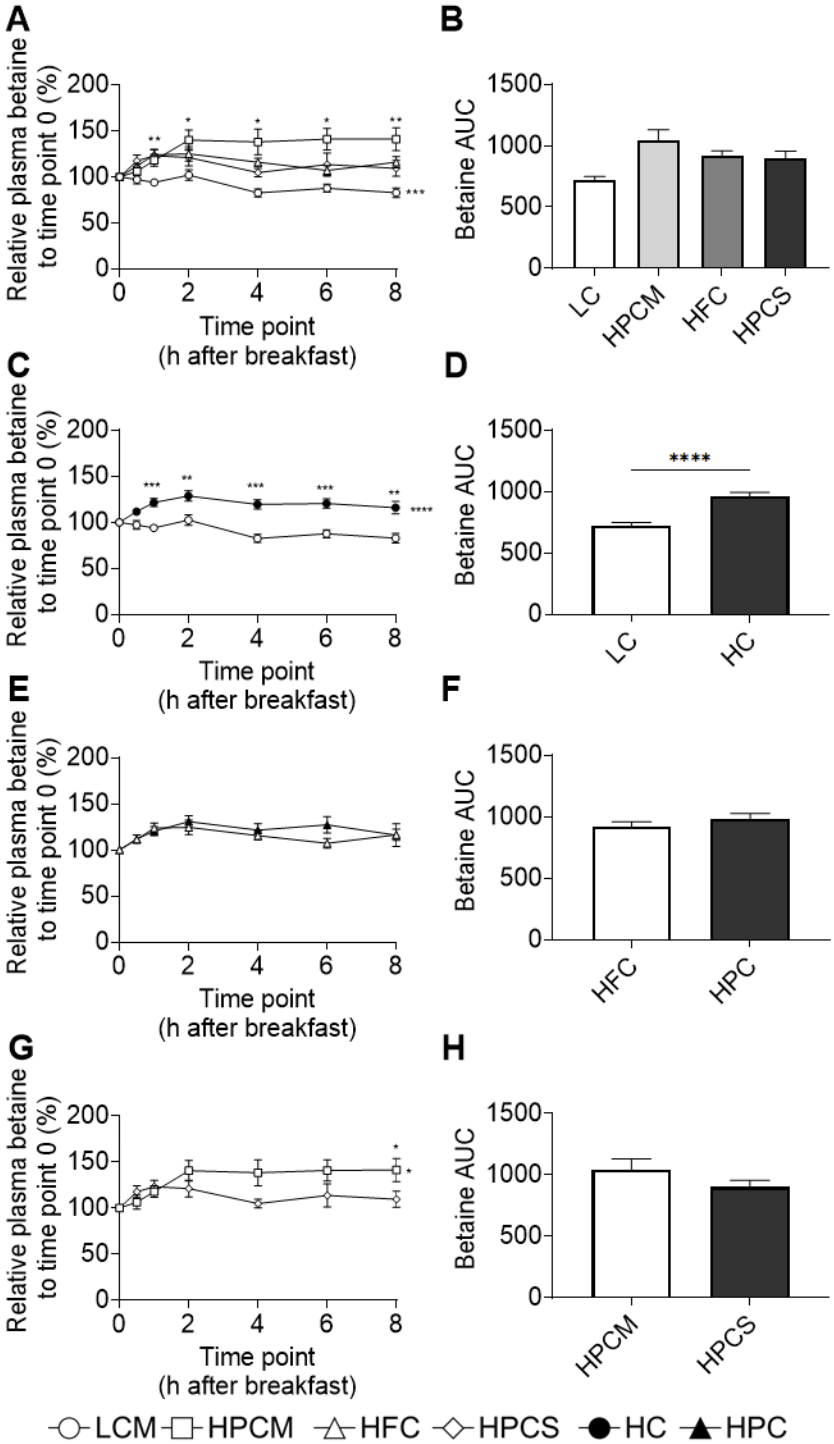
Postprandial plasma TMAO from type of breakfast and AUC, n=8 (A, B), amount of choline grouped breakfasts and AUC, n=8/24 (C, D), type of choline grouped breakfasts and AUC, n=8/16 (E, F), and source of PC breakfasts and AUC, n=8 (G, H). Data are presented as mean ± SEM, n = 8-24. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Low choline, LCM; high PC meal, HPCM; high free-choline, HFC; high PC from supplement, HPCS; high choline, HC; high PC, HPC; trimethylamine N-oxide, TMAO.

Figure 4.3Postprandial plasma choline response



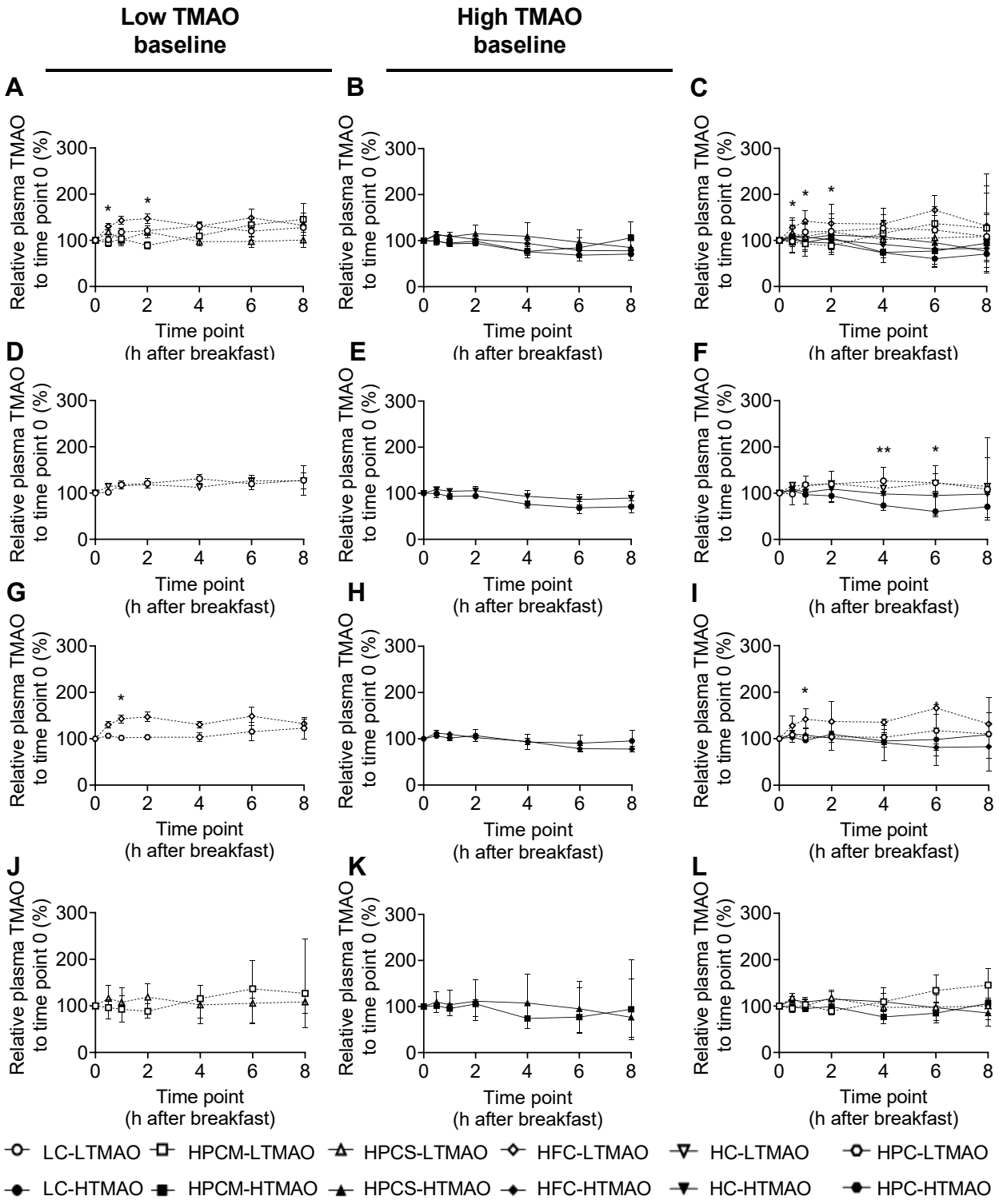
Postprandial plasma choline from type of breakfast and AUC, n=8 (A, B), amount of choline grouped breakfasts and AUC, n=8/24 (C, D), type of choline grouped breakfasts and AUC, n=8/16 (E, F), and source of PC breakfasts and AUC, n=8 (G, H). Data are presented as mean ± SEM, n = 8-24. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Low choline, LCM; high PC meal, HPCM; high free-choline, HFC; high PC from supplement, HPCS; high choline, HC; high PC, HPC.

Figure 4.4Postprandial plasma betaine response



Postprandial plasma betaine from type of breakfast and AUC, n=8 (A, B), amount of choline grouped breakfasts and AUC, n=8/24 (C, D), type of choline grouped breakfasts and AUC, n=8/16 (E, F), and source of PC breakfasts and AUC, n=8 (G, H). Data are presented as mean ± SEM, n = 8-24. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Low choline, LCM; high PC meal, HPCM; high free-choline, HFC; high PC from supplement, HPCS; high choline, HC; high PC, HPC.

Figure 4.5Postprandial plasma TMAO response in Low and High TMAO baseline groups



Postprandial plasma TMAO from type of breakfast and low/high TMAO baseline group, n=4 (A-C), amount of choline grouped breakfasts and low/high TMAO baseline group, n=4/12 (D-F), type of choline grouped breakfasts and low/high TMAO baseline group, n=4/8 (G-I), and source of PC and breakfasts low/high TMAO baseline group, n=4 (J-L). Data are presented as mean ± SEM or medians [95% CIs], n = 4-12. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Low choline in the low TMAO baseline group, LC-LTMAO; Low choline in the high

TMAO baseline group, LC-HTMAO; high choline in the low TMAO baseline group, HC-LTMAO; high choline in the high TMAO baseline group, HC-HTMAO; high free-choline in the low TMAO baseline group, HFC-LTMAO; high free-choline in the high TMAO baseline group, HFC-HTMAO; high PC in the low TMAO baseline group, HPC-LTMAO; high PC in the high TMAO baseline group, HPC-HTMAO; high PC meal in the low TMAO baseline group, HPCM-LTMAO; high PC meal in the high TMAO baseline group, HPCM-HTMAO; high PC from supplement in the low TMAO baseline group, HPCS-LTMAO; high PC from supplement in the high TMAO baseline group, HPCS-HTMAO.

4.4 Discussion

Choline is an essential nutrient (Institute of Medicine 1998; Zeisel et al. 1991; Zeisel and da Costa 2009). Since the beginning of the last decade several studies have proposed that high choline diet leads to CVD risk by elevating plasma TMAO (Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; Lever et al. 2014; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; W.H. Wilson Tang et al. 2015; W. H. W. Tang et al. 2015; Wang et al. 2011, 2014; Zhu et al. 2016). However, an number of recent animals studies have shown that high choline diets do not increase atherosclerosis despite elevating plasma TMAO levels (Aldana-Hernández et al. 2020; Lindskog Jonsson et al. 2018). In the same context, studies in humans have reported that dietary choline is not associated to CVD risk (Meyer and Shea 2017). The aim of the present study is to determine the acute effect of different dietary forms and sources of choline on postprandial choline and TMAO metabolism in healthy men. Interestingly plasma TMA and TMAO levels postprandial responses did not differ among the four breakfasts. Recent dietary interventions have studied the impact of choline-containing meals in plasma TMAO, unfortunately, plasma TMA was not measured in most of these studies. DiMarco, et al. performed a crossover dietary intervention where d 38 healthy participants (16 men and 16 women) were recruited and underwent for a 2-week washout period of no egg consumption (324 mg of choline/day intake) followed by the consumption of 1,2, and 3 eggs/day for 4 weeks (421, 563, and 696 mg of choline/day, respectively). Participants were instructed to abstain of consume more eggs than indicated and any main egg-containing food. The study reported that plasma TMAO levels were not altered by consuming up to 3 eggs/day for 4 wks (DiMarco et al. 2017). In a cross over study conducted by Missimer, et al., 50 healthy participants (24 women and 26 men) consumed 2 eggs or one packet of oatmeal as first meal of

the day for 4 weeks. Participants could add anything they wanted (vegetables, meat, cheese, syrup, yogurt, etc.) and prepare the breakfast as they desired (Missimer et al. 2017, 2018). As a secondary outcome from the initial study (Missimer et al. 2017), plasma TMAO levels did not change with the dietary intervention (Missimer et al. 2018). In a recent randomized crossover study, 37 men received control (low choline), choline bitartrate, or soy PC supplemented meal (42, 619, and 623 mg of total choline, respectively) with 1-week washout period in between. After 4 h of receiving the choline bitartrate supplemented meal, plasma TMAO concentration was significantly higher compared to the no choline control and the soy PC meals (Cho et al. 2020). Mödinger, et al. (2019) investigated the kinetics of choline and the generation of choline metabolites from two different choline sources. Twelve participants took a single choline bitartrate (620 mg of choline) or krill oil capsule (PC, 572 mg of choline and 1840 mg of EPA and DHA) with 14 days washout period. Six participants received a single dose of fish oil (1800 mg of EPA and DHA, no choline) as a control. Plasma TMAO levels were significantly elevated after 8 h of having both choline interventions (the choline bitartrate capsule and the krill oil capsule) and reached a peak after 12 h; however, TMAO was higher with choline bitartrate than the krill oil capsule (Mödinger et al. 2019).The amount of total choline used in these studies varied. Those where eggs were used did not report the choline content from the actual meals studied. The main difference in the experimental design between these studies and ours is that the meals were not well balanced for fibre, protein, and fat content. A recent study reported that mice fed with high amylose resistant starch had higher plasma and fecal TMA and TMAO concentrations compared to mice fed a native wheat starch (Koay et al. 2020). The high amylose resistant starch fibre is most present in the diet, such whole grains, legumes, cooked and chilled pasta, potatoes and rice, and unripe bananas. This study suggests that the type of the fibre from diet might drive the gut microbiota involved in TMA

and TMAO production (Koay et al. 2020). In order to not alter the TMA production, the meals designed in our study were balanced for amount and type of fibre (*Table 4.1*). However, the distribution of the intestinal microbiome was not measured.

Looking on the 24 h recalls of the participants (*Table 4.4*), the average choline intake was below the AI. Although dietary intake varied among participants. Cassambai et al, (2019) studied the effect of acute choline bitartrate dose (700 mg) in 18 healthy volunteers. The postprandial response of plasma TMAO levels varied amongst participants. For the statistical analysis participants were divided into high and low TMAO groups based on the baseline TMAO levels. When urine samples were analyzed, it was reported that individuals in the high TMAO group had a significantly higher response compared to those in the low TMAO group after 4 h of taking the choline capsule. The dietary records from 2 wks showed that TMA rich food intake (meat, egg, and fish) was similar between the groups, unfortunately choline moieties intake was not reported. Interestingly, this study proposes the terms TMAO responders and TMAO non-responders to individuals respond unequally to acute choline dose intake (Cassambai et al. 2019). A cross-sectional cohort in 297 healthy participants reported that those in the highest plasma TMAO quartile had the highest choline intake (Krüger et al. 2017). Consistently, a recent study reported a positive correlation between choline intake and plasma TMAO levels in 1981 patients who underwent for an elective coronary angiography (Van Parys et al. 2020) Together, these and ours results might indicate long-term intake of dietary choline likely plays a more prominent role in increasing plasma TMAO levels versus a single meal.

Traditionally, plasma choline level has been used as a marker for choline intake; however, it appears to be unreliable (Abratte et al. 2009). In the present acute study postprandial plasma choline response was unchanged among the four breakfast. Even though recent cross over dietary

interventions with high choline meals have shown to increase on plasma choline levels (Lemos et al. 2018; Miller et al. 2014; Missimer et al. 2018; Wallace et al. 2012; Zhu et al. 2020). This difference might be due to the duration of the dietary intervention suggesting that a long-term high choline diet leads to increased plasma choline levels.

Betaine is the product of the choline oxidation in the mitochondria in the liver and serves as methyl donor group (Ueland 2011). Plasma betaine levels were elevated in the three high choline breakfast after one hour mainly due to those meals were also high in betaine content. An acute high dose of betaine increased plasma betaine levels within hours (Schwahn et al. 2003). As well, it has been showed that long term of consumption of either betaine rich diet or betaine supplement elevates plasma betaine levels within 3 days (Atkinson et al. 2009). Also, long term high dietary choline results in plasma betaine levels also being elevated (Wallace et al. 2012; Zhu et al. 2020).

This study had some limitations, including the low number of participants. Participants consumed their regular diet before each visit to the research clinic thus we did not control for the consumption of TMAO-precursors foods. To analyze the dietary intake of macro and micro nutriments we used 24 h recalls. Unfortunately, we did not collect urine samples to determine the type of TMAO response among the participants. Another limitation was that we did not measure the intestinal microbiota population nor distribution.

In summary, consumption of a high choline (~360 mg of choline) meal did not impact postprandial TMAO response in healthy men. However, plasma TMAO levels were increased after 0.5 and 1 h of consuming a high free-choline breakfast compared to high PC breakfasts. Interestingly, we found that the consumption of HPCM elevates plasma betaine response compared to HPCS meal consumption. This suggests that the PC from the meal is metabolized to choline and then might be rapidly absorbed and get the liver where it is oxidized to betaine. The current evidence shows that

the effect of diet on TMAO metabolism in humans is a complex process. Choline supplemented meals (above AI) might increase post-prandial TMAO response. However, long-term effects of dietary choline versus PC supplementation on TMAO levels need further examination. Limiting choline intake bellow the AI due to the impact on TMAO production might be unwise.

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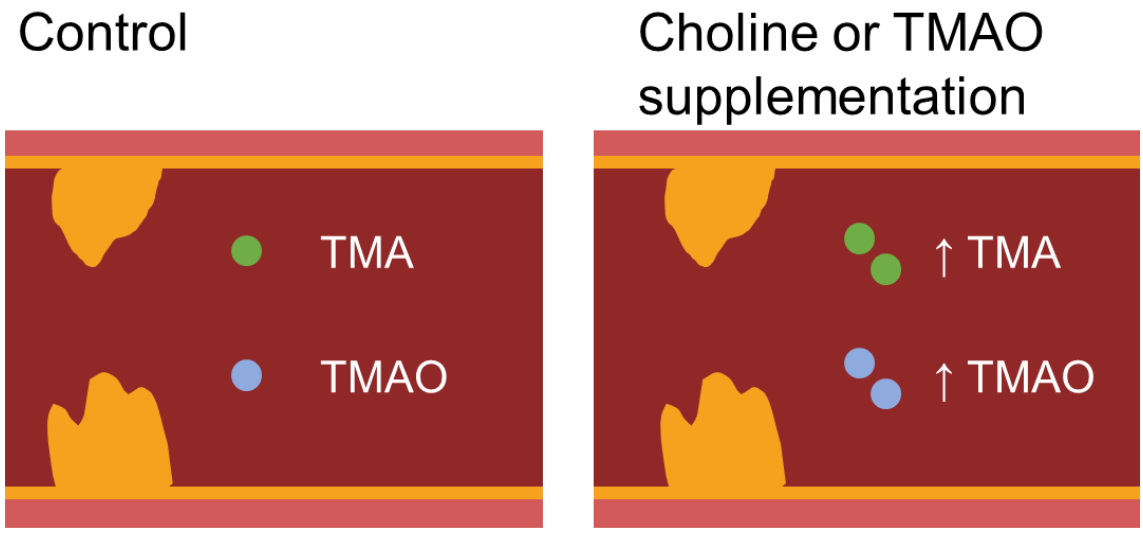
Chapter 5: Final discussion

5.1 Executive summary of findings

5.1.1 Dietary choline or trimethylamine N-oxide supplementation does not influence atherosclerosis development in *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ male mice

In chapter 2 we investigated how dietary supplementation of choline-related metabolites (10X normal choline, betaine, and TMAO) influences atherosclerosis development in 2 atherogenic mouse models *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$) mice. We reported that in *Ldlr*$^{-/-}$ mice, dietary supplementation for 8 wk with choline or TMAO increased plasma TMAO concentrations by 1.6- and 4-fold, respectively. After 16 wk, there was a 2-fold increase in plasma TMAO after dietary TMAO supplementation. In *Apoe*$^{-/-}$ mice, dietary supplementation with choline, betaine, or TMAO for 12 wk did not increase plasma TMAO concentrations. However, choline and TMAO supplementation for 28 wk significantly increased plasma TMAO concentrations by 1.8- and 1.5-fold, respectively. Contrary to predictions, atherosclerotic lesion size was not altered by any of the dietary interventions, regardless of mouse model (*Figure 5.1*).

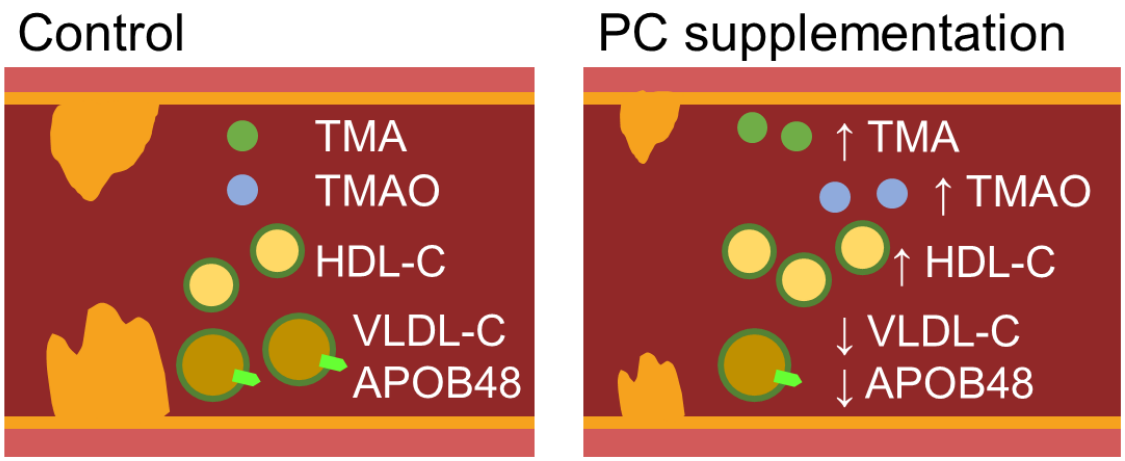
Figure 5.1 Dietary choline or TMAO supplementation does not influence atherosclerosis



5.1.2 Dietary phosphatidylcholine supplementation reduces atherosclerosis in *Ldlr*$^{-/-}$ male mice

In Chapter 3 we investigated the effect of the type of choline, free-choline (0.4%, 4 times normal) or PC (0.1% free- choline and 0.3% choline from PC), on atherosclerosis development. We found that PC-supplemented *Ldlr*$^{-/-}$ male mice had significantly lower atherosclerotic lesions despite having 2-fold higher plasma TMAO levels compared to both control and choline-supplemented groups. We found that PC supplementation decreased plasma very low-density lipoprotein-cholesterol (VLDL-C) and APOB48, and increased plasma high-density lipoprotein-cholesterol (HDL-C). However, VLDL secretion was not affected by dietary treatment. We also observed lower levels of circulating pro-atherogenic chemokines in the PC-supplemented group. This study suggests that increased dietary PC intake does not induce a pro-atherogenic phenotype (*Figure 5.2*).

Figure 5.2 Dietary PC supplementation reduces atherosclerosis in Ldlr$^{-/-}$ *male mice*



5.1.3 Examining the acute role of different dietary sources of choline on postprandial choline and TMAO metabolism in humans: a randomized controlled crossover trial

The objective of Chapter 4 was to examine the effect of standardized breakfasts containing different dietary forms and sources of choline on postprandial choline and TMAO metabolism in healthy men. We observed that consumption of a high choline (~360 mg of choline) meal did not impact the overall postprandial (8 hours after meal) TMAO response in healthy men. However, plasma TMAO levels were transiently increased at 0.5 and 1 h after consuming a high free-choline breakfast compared to high PC breakfasts. High choline meals also contained more betaine; therefore it was not surprising that appearance of betaine into circulating was increased following consumption of these meals. Interestingly, we found that the consumption of high PC meal increased plasma betaine compared to high PC supplemented meal. Clearly the effect of diet on TMAO metabolism in humans is a complex process. The studies from this thesis provides strong evidence that the amount, type, and source of dietary choline supplementation.

5.2 General discussion and future directions

It has been established that choline is required for several biological processes (Institute of Medicine 1998; Zeisel et al. 1991; Zeisel and da Costa 2009). Choline could be synthesized *de novo* biosynthesis through three consecutive methylation of PE to PC in the liver (Vance 2014). However, *de novo* choline synthesis is not enough to cover the human needs and it is required to be included in the diet. To date the AI is 550 and 425 mg/d for adult men and women, respectively (Institute of Medicine 1998). Free-choline and PC are two of the three most common forms of choline in the diet of North American population (Lewis, Subhan, et al. 2014; Yonemori et al. 2013). In order to meet the AI it has been recommended to increase the consumption of choline rich foods like eggs, dairy, meat, soy bean, and broccoli (high in PC) (Patterson et al. 2008b).

Another important source of choline are vegetable, fruit, cereal and some legumes, which are rich in FC (Patterson et al. 2008b). Regardless, recent studies have hypothesized that consumption of choline rich foods (specifically PC from animal sources) may increase cardiovascular disease (CVD) risk through elevating circulating TMAO concentrations (Haghikia et al. 2018; Hartiala et al. 2014; Koeth et al. 2013, 2014; de la Huerga and Popper 1951; Lever et al. 2014; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; Tang et al. 2015; Tang et al. 2015b; Wang et al. 2011, 2014; Zeisel 1981; Zhu et al. 2016). In order to understand the mechanisms by which dietary PC enhances atherosclerosis several dietary trials in animals have been completed; however, most of the animal studies have used FC (choline bitartrate or choline chloride), but not PC as the choline source (Gregory et al. 2015; Haghikia et al. 2018; Hartiala et al. 2014; Huc et al. 2018; Koeth et al. 2013, 2014; Lever et al. 2014; Lindskog Jonsson et al. 2018; Mafune et al. 2016; Senthong, Li, et al. 2016; Senthong, Wang, et al. 2016; Suzuki, Liam M. Heaney, et al. 2017; Tang et al. 2013, 2014; Tang et al. 2015; Tang et al. 2015b; Wang et al. 2011, 2014; Zhu et al. 2016). It has reported that FC and PC absorption is identical in rats (Cheng et al. 1996).The research outlined in this thesis clearly shows that the type of dietary choline impacts atherosclerosis development, regardless of TMAO levels. Moreover, we reported that PC reduces atherosclerosis, suggesting that the effect of PC on plaque formation is independent of microbiome-dependent production of TMA. Additionally, dietary PC decreased plasma very low-density lipoprotein-cholesterol (VLDL-C) and apolipoprotein B48 (APOB48), and increased plasma high-density lipoprotein-cholesterol (HDL- C). These findings suggest that PC might influence the clearance process of the atherosclerotic plaque. The mechanisms remain unclear; however, the phospholipid composition of lipoproteins modulate the removal from the plasma (Zhao et al. 2009). The immune system

plays a crucial role in the clearance of atherosclerotic plaque. Our research group has shown that PC improves immune development and function in animal trials (Dellschaft et al. 2018; Lewis et al. 2017; Lewis, Richard, et al. 2015; Richard, Lewis, et al. 2017). Further investigation is needed to understand how PC improves atherosclerosis progression.

There have been several dietary interventions in humans showing that foods rich in choline, or TMAO increase postprandial plasma TMAO levels (Cho et al. 2017, 2020; Hagen et al. 2020; Miller et al. 2014). In these reports, other components of the meal were not standardized. Therefore, we wanted to investigate the impact of dietary choline in meals where the amount of energy, fat and fibre were controlled. We also choose choline levels that could be reasonable achieved in a normal meal. Although our data was inconclusive, our human trial was properly designed and well controlled. Further research is needed to understand the long-term of dietary choline supplementation on atherosclerosis development in humans.

5.2.1 Dietary choline, PC and TMAO increases TMAO in atherogenic mouse models

The results from this thesis (Chapter 2 and 3) have provided evidence that diets supplemented with choline, PC, and TMAO increases plasma TMAO levels in *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ male mice. However, atherosclerosis development was not enhanced by any of the dietary intervention. In mice, plasma TMAO levels are typically ~1 to 10 μM under normal conditions. It has been observed that dietary choline and TMAO supplementation increased plasma TMAO levels (>20 μM) and enhanced atherosclerosis development (Koeth et al. 2013; Seldin et al. 2016; Wang et al. 2011; Yang et al. 2019). In agreement with our findings, others have reported a beneficial or neutral effect of choline supplementation or TMAO treatment in atherosclerosis and blood pressure, despite elevating plasma TMAO levels (<20 μM) (Gao et al. 2014; Huc et al. 2018; Koay et al. 2020; Lindskog Jonsson et al. 2018; Shih et al. 2015). In our studies, we only observed a small increase (1-2 fold)

in plasma TMAO levels, while others reported a 50-fold increase. It is possible that a large increase in plasma TMAO is required to induce atherosclerosis development. It should be pointed out that our data does not directly contradict the data from other groups. In our mice, 3x choline/PC supplementation does not increase plasma TMAO levels to a level that may induce atherosclerosis. It should also be pointed out that a number of labs have recently reported a lack of correlation between dietary choline supplementation, TMAO levels and atherosclerosis (Collins et al. 2016; Lindskog Jonsson et al. 2018). Our study adds data to this important biological topic. Therefore, the effects of dietary choline on atherosclerosis development may depend on housing facilities, age, and the source or form of dietary choline supplementation. For example, the TMAO we used was 99% purity compared to the 67% that was used by Wang et al. (Wang et al. 2011). It is also possible that the microbiome of the colony is likely very important factor in determining the relationship between dietary choline and TMAO. Some studies use long-term oral antibiotics to test this relationship; however, we showed that PC supplementation reduces atherosclerosis, despite an increase in plasma TMAO, suggesting that the effect of PC on plaque formation is independent of microbiome-dependent production of TMA. It is more likely that mice used in this study did not have sufficient TMA-producing microbes to induce TMAO production to a level that might have induced atherosclerosis. Further investigation of the effect of dietary PC supplementation on the TMA-lyase bacteria population and distribution focusing on those that encoded the Cut gene clusters like *Firmicutes Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Lentisphaerae*.

Dietary choline supplementation experiments in mice have used free-choline (choline bitartrate or choline chloride) (Gregory et al. 2015; Lindskog Jonsson et al. 2018; Wang et al. 2011; Zhu et al. 2016). Chapter 3 of this thesis showed that PC supplementation reduces atherosclerosis

development, despite elevating plasma TMAO levels. These observations are consistent with studies that observed that PC administration (orally or directly into the stomach) reduced atherosclerosis in miniature pigs and white rats by reducing serum levels of total lipids, TG, cholesterol, and APOB (Samochowiec, Kadłubowska, Różewicka, et al. 1976; Samochowiec, Kadłubowska, and Różwicka 1976). Unlike our experiments, these prior studies did not control for the choline or fatty acid content of the diet, two variables that would affect atherosclerosis.

5.2.2 Differential effects of choline forms on TMAO production in humans

In chapter 4 the effect of 4 meals with different dietary forms, sources, and amount of choline on plasma TMAO response was examined in a randomized controlled crossover trial in healthy men. We observed that plasma TMAO response remain similar among the tested meals. However, the postprandial response on plasma TMAO levels varied amongst participants. Some studies have observed that up to daily consumption of three eggs (rich in PC) might not be associated with CVD risk factors (DiMarco et al. 2017; Miller et al. 2014; Missimer et al. 2018). The consumption of foods rich in choline, carnitine, or TMAO content (such as eggs, fish) have shown an increase postprandial plasma TMAO levels (Cho et al. 2017, 2020; Hagen et al. 2020; Miller et al. 2014). Nevertheless, those diets were not standardized for energy, fat or fibre content. It has observed that that mice feed with high amylose resistant starch had higher plasma and fecal TMA and TMAO levels compared to a native starch (Koay et al. 2020). This study provides evidence that fibre from diet drives the gut microbiota variety involved in TMA and TMAO production (Koay et al. 2020). Another aspect interesting to contemplate is the identification of FMO3 variants that might contribute to the high variability of the TMAO response. Several factors contribute to the variability of TMAO response that need further investigation such as background diet, gut microbiome, and genotype.

5.3 Limitations

Studies from chapters 2 and 3 have some limitations. 1) Only males were used, even though the relation of TMAO and atherosclerosis has been reported in both females and males (Wang et al. 2011). It is very possible that female mice metabolize PC differently than male mice as have a higher rate of choline synthesis as compared to males (Noga and Vance 2003a). 2) The cholesterol content of the HFD was high (0.5%) which could impact any potential effect of dietary choline on atherosclerosis. 3) Atherosclerosis lesions were measured only in the aortic root and not in multiple regions of the aortic branch. 4) The soy lecithin supplement used contained 23% PC. Although supplemented diets were matched for choline content, possibly other components may contribute to the biological consequences. 5) Fatty acids composition of the meals was modified to control meal (*Table 3.2*): however, we are aware that PCS diet had slightly higher total n-3 fatty acids. It has been reported that 3-n fatty acids treatment might reduce CVD risk (Perez-Martinez, Katsiki, and Mikhailidis 2020). 6) Only one source of PC (soy lecithin) was used in our study. Interestingly, Balb/c mice treated by oral gavage with an emulsion of PC from soy lecithin had higher plasma TMA and TMAO than those with emulsions of PC from egg yolk or from squid (Gao, Jiang, et al. 2016). 7) Even though the diets were balanced for the choline content from PC, the amount of other phospholipids were not. Phospholipid remodeling influences atherosclerosis development (Linton et al. 2000). Indeed, in the intestine, lsyoPC is needed for intracellular lipid trafficking and chylomicron biosynthesis (Hui 2016) and sphingomyelin treatment inhibits absorption of cholesterol (Cohn et al. 2010). This may be explained by a reduction in absorption rate of soy PC versus other sources; however, the mechanisms for this observation have not been explained (Küllenberg et al. 2012).

Chapter 4 has limitations. 1) the sample size was small; however, half of the participants had high TMAO levels at baseline (median=1.75 μM, CI: 0.98 – 6.18). 2) The variability on the overall TMAO response among the participants. 3) Participants consumed their regular diet before each visit to the research clinic thus the consumption of TMAO-precursor foods was not controlled. 4) Identification of gut microbiota was not determined. 5) Urine samples were not collected to determine the type of TMAO response among the participants. 6) The soy lecithin supplement used contains 23% PC. Despite the high choline meals were designed to have the same amount of choline, there is a possibility that other ingredients may affect the response. 7) Non-atherosclerosis biomarkers were measured.

5.4 Conclusion

The initial purpose of this research was to investigate first at which amount of choline supplementation atherosclerosis was enhanced and the mechanisms behind it. Regardless, after several animal experiments, we did not observe these phenomena. Collectively, the studies in this thesis confirmed that high choline intake elevates plasma TMAO levels. However, from the 2 studies conducted in atherogenic mouse models, the elevation on plasma TMAO levels did not enhance atherosclerosis development. We further observed that dietary PC supplementation decreased atherosclerotic lesion size. These findings provide evidence that the type of choline influences atherosclerosis progression. The cross-over trial showed that a single high choline meal did not alter the overall postprandial TMAO response. This thesis provides evidence that high levels of choline and PC supplemented diets increase plasma TMAO levels in mice. However, the plasma TMAO levels were not high enough to enhance atherosclerosis. Even though PC supplemented-diet reduced atherosclerotic plaque size. In humans, high free-choline supplemented

meals (above AI) seem to increase post-prandial TMAO response. Recommending to limit choline intake below the AI due to the impact on TMAO production might be unwise.

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Appendix

Appendix 1:Standard Operating Procedures for the preparation of the low choline meal - Choline Breakfast Study for the study in Chapter 4

Scope: To make Low choline meal for Choline Breakfast Study

a) Materials/Ingredients:

Product/Ingredient Name	Company/Brand	Amount
Complete pancake mix, dry	Aunt Jemima	33 g
Egg whites, raw	Simply	210 g
Butter, salted	Foremost	29 g
Blueberries, raw	Superstore	15 g
Fibre powder supplement		10 g
Parchment paper		2 squares (~5" x 5")
Electronic kitchen scale		1
Small bowl		2
Non-stick frying pan		1
Spatula		2
Fork		2
Knife		1
Plate		1
Serving tray		1

Training Required: HNRU kitchen usage training is required by all personnel.

Control/Protective Measures: Lab coats, gloves

b) Step by Step Procedure of the Process:

1. Place plate on serving tray.
2. Place bowl on scale and tare scale.
3. Weigh 33 g of dry pancake mix and tare scale.
4. Weigh 10 g of powdered fibre supplement and tare scale again.
5. Weigh 50 g of water and use a fork to mix pancake batter thoroughly until no lumps remain, and is a thick paste. Can add more water is necessary.
6. Place sheet of parchment paper on scale and tare scale.
7. Weigh 15 g of blueberries and add to pancake batter. Set pancake batter aside.

8. Place sheet of parchment paper on scale and tare scale.
9. Weigh 29 g of butter.
10. Add approximately half of the weighed butter to frying pan on medium heat.
11. Once butter is melted, add pancake mixture to frying pan and cook until bubbles appear and bottom turns golden brown (approximately 2 minutes).
12. Flip pancake with spatula and cook another 2 minutes until remaining side is brown. Add pancake to plate on serving tray.
13. Using another bowl, place bowl on scale and tare scale.
14. Weigh 210 g of egg whites. Set aside.
15. Add the remaining half of the weight butter to frying pan on medium heat.
16. Add egg whites to the frying pan and use spatula to move the egg whites until no longer translucent and cooked through.
17. Add cooked egg whites to plate on serving tray.
18. Add fork and knife to serving tray and bring tray to participant.

Appendix 2: Standard Operating Procedures for the preparation of the high free-choline meal - Choline Breakfast Study for the study in Chapter 4

Scope: To make high free-choline meal for Choline Breakfast Study

a) Materials/Ingredients:

Product/Ingredient Name	Company/Brand	Amount
Cheddar cheese, medium	Armstrong	20 g
Egg whites, raw	Simply	60 g
Whole wheat bread	Old Mill	30 g
Peanut butter, smooth	Kraft	16 g
Skim milk	Lucerne	90 g
Clementine, raw, sections		40
Pistachios, dry roasted, shelled	President's Choice	19 g
Fibre powder supplement		5.6 g
Choline bitartrate	Trophic	3 tablets
Parchment paper		1 square (~5" x 5")
Electronic kitchen scale		1
Cheese grater		1
Small bowl		1
Non-stick frying pan		1
Spatula		1
Toaster		1
Fork		2
Knife		2
Plate		1
Glasses		2
Serving tray		1

Training Required: HNRU kitchen usage training is required by all personnel.

Control/Protective Measures: Lab coats, gloves

b) Step by Step Procedure of the Process:

1. Place plate on serving tray.
2. Use cheese grater to grate cheddar cheese.
3. Place sheet of parchment paper on electronic kitchen scale and tare scale.
4. Weigh 20 g of cheddar cheese, set aside.
5. Place bowl on scale and tare scale.

6. Weigh 60 g of egg whites, set aside.
7. Place sheet of parchment paper on scale and tare scale.
8. Weigh 5.6 g of powdered fibre and add to add whites. Use fork to mix thoroughly into egg whites.
9. Over medium heat, add egg whites to frying pan and cook 1 minute, moving cooked egg into the middle of the pan.
10. Add cheddar cheese to egg whites and cook until egg whites are no longer translucent, then add to plate on the serving tray.
11. Meanwhile, weigh 30 g of bread on scale and place in toaster.
12. Place sheet of parchment paper on scale and tare scale.
13. Weigh 16 g of peanut butter.
14. Once bread is toasted, spread peanut butter on toast and add to plate on the serving tray.
15. Shell pistachios and weigh 19 g of shelled pistachios on scale.
16. Add pistachios to plate on the serving tray.
17. Peel clementine.
18. Weigh 40g of clementine sections and place on serving tray.
19. Place glass on scale and tare scale.
20. Weigh 90 g of skim milk and place glass on serving tray.
21. Fill additional glass with water and place on serving tray.
22. Take one choline bitartrate tablet and place on serving tray.
23. Add fork and knife to serving tray and bring tray to participant.

Appendix 3: Standard Operating Procedures for the preparation of the high PC meal - Choline Breakfast Study for the study in Chapter 4

Scope: To make high PC meal for Choline Breakfast Study

a) Materials/Ingredients:

Product/Ingredient Name	Company/Brand	Amount
Whole eggs, raw	No Name, Grade A Extra Large	130 g
Egg whites, raw	Simply	30 g
Bacon, cooked	PC Free From	13 g
Whole wheat bread	Old Mill	30 g
Butter, salted	Foremost	7 g
Blackberries, raw	Superstore	75 g
Raspberries, raw	Superstore	40 g
Fibre powder supplement		2.7 g
Parchment paper		1 square (~5" x 5")
Electronic kitchen scale		1
Small bowl		3
Non-stick frying pan		1
Spatula		1
Toaster		1
Fork		2
Knife		2
Plate		1
Glasses		2
Serving tray		1

Training Required: HNRU kitchen usage training is required by all personnel.

Control/Protective Measures: Lab coats, gloves

b) Step by Step Procedure of the Process:

1. Start heating frying pan over medium heat.
2. Place plate on serving tray.
3. Place sheet of parchment paper on scale and tare scale.
4. Weigh 7 g of butter. Add half to frying pan, and reserve other half for toast.
5. In a bowl, crack eggs and use fork to whisk eggs.
6. Using a separate bowl, place bowl on scale and tare scale.

7. Weigh 130 g of whisked egg and tare scale.
8. Weigh 30 g of egg whites and tare scale.
9. Weigh 2.7 g of powdered to fibre and use a fork to mix thoroughly.
10. Add egg fibre mixture to frying pan and use spatula to move eggs around until cooked through.
11. After eggs are cooked, add to plate on serving tray.
12. Using the same frying pan over medium heat, add 2-3 slices of bacon and cook until crispy.
13. Place sheet of parchment paper on scale and tare scale.
14. Weigh 13 g of cooked bacon and add to plate on serving tray.
15. Meanwhile, weigh 30 g of bread on scale and place in toaster.
16. Once bread is toasted, spread remaining half of reserved butter on toast and add to plate on the serving tray.
17. Place bowl on scale and tare scale.
18. Weigh 75 g of blackberries and tare scale again.
19. Weigh 40 g of raspberries.
20. Add berries to plate on serving tray.
21. Add fork and knife to serving tray and bring tray to participant.

Appendix 4: Standard Operating Procedures for the preparation of the high PC supplemented meal - Choline Breakfast Study for the study in Chapter 4

Scope: To make high PC supplemented meal for Choline Breakfast Study

a) Materials/Ingredients:

Product/Ingredient Name	Company/Brand	Amount
Whole almonds	Trophy Natural Almonds	36 g
Egg whites, raw	Simply	120 g
Olive oil	No Name	2 g
Spinach, cooked	No Name, Frozen	25 g
Red pepper, cooked	Superstore	75 g
Green peas, cooked	Non Name, Frozen	60 g
Pear, raw	Superstore, Bartlett	75 g
Soy lecithin granules	GNC	1.25 tbsp (9.46 g)
Parchment paper		1 square (~5" x 5")
Electronic kitchen scale		1
Pot		1
Plastic storage containers		3
Cutting board		1
Small bowl		2
Non-stick frying pan		1
Spatula		1
Fork		2
Knife		2
Plate		1
Serving tray		1

Training Required: HNRU kitchen usage training is required by all personnel.

Control/Protective Measures: Lab coats, gloves

b) Step by Step Procedure of the Process:

1. In amicrowave safe bowl, defrost spinach. Repeat with frozen green peas.
2. Place in separate plastic storage containers and place into fridge for further use.
3. Use cutting board and knife to dice red pepper. Place diced red pepper into frying pan and sauté until soft.
4. Place in plastic storage containers and place into fridge for further use.

5. Place plate on serving tray.
6. Place sheet of parchment paper on scale and tare scale.
7. Weigh 60 g of almonds and add to plate on serving tray.
8. Place bowl on scale and tare scale.
9. Weigh 120 g of egg whites and tare scale.
10. Weigh 9.46 g of soy lecithin granules and use a fork to mix thoroughly into egg whites. Set aside.
11. Using another bowl, place bowl on scale and tare scale.
12. Weigh 25 g of cooked spinach from plastic container and tare scale.
13. Weigh 75 g of cooked red pepper from plastic container and tare scale.
14. Weigh 60 g of cooked green peas from plastic container and tare scale.
15. Place frying pan on scale and tare scale.
16. Weigh 2 g of olive oil.
17. Heat frying pan with oil over medium heat.
18. Add egg and soy lecithin mixture to frying pan and cook for approximately 30 seconds.
19. Add vegetable mixture to frying pan and use spatula to move the egg mixture around until cooked through.
20. After egg mixture is cooked, add to plate on serving tray.
21. Using cutting board and knife to cut pear into slices.
22. Place sheet of parchment paper on scale and tare scale.
23. Weigh 75 g of sliced pear and add to plate on serving tray.
24. Add fork and knife to serving tray and bring tray to participant.

Appendix 5: Standard Operating Procedures for the preparation of the low choline snack - Choline Breakfast Study for the study in Chapter 4

Scope: To make low choline snack for Choline Breakfast Study

a) Materials/Ingredients:

Product/Ingredient Name	Company/Brand	Amount
Apple, raw	Superstore, Gala	150 g
Yogurt	Foremost, Stirred Blueberry (1% MF)	120 g
Parchment paper (approx 5" x 5")		1 square
Electronic kitchen scale		1
Cutting board		1
Knife		1
Small bowl		1
Plate		1
Serving tray		1

Training Required: HNRU kitchen usage training is required by all personnel.

Control/Protective Measures: Lab coats, gloves

b) Step by Step Procedure of the Process:

1. Place plate on serving tray.
2. Using cutting board and knife to cut apple into slices.
3. Place sheet of parchment paper on scale and tare scale.
4. Weigh 150 g of sliced pear and add to plate on serving tray.
5. Place bowl on scale and tare scale.
6. Weigh 120 g of yogurt and put bowl on plate on serving tray.
1. Add fork and knife to serving tray and bring tray to participant.

Appendix 6: Standard Operating Procedures for use of the University of Alberta Database for the Choline Content of Common Foods (Alberta Database) (Lewis 2016)

Excel worksheets contains the Alberta Database. The Alberta Database was designed to estimate the choline intake by women in the APrON cohort, however it can be applied to any population. The figures shown in the following sections are from the Alberta Database and are included to serve as a guide for further understanding of the database.

a) Choline Database Worksheet

The first worksheet in the Alberta Database is Choline Database that includes the choline content of all foods in the database. This database is used to estimate dietary choline intake. *Figure A.1* is a fragment of the Choline Database worksheet.

Figure A.1. Choline Database worksheet

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Assigned NDB	Food Processor Description	Assigned NDB	USDA Description	Food Category	Dairy or Eggs	Betaine	Free Cho	GPC	Pch	Ptdcho	SM	Total Choline	a.NDB
2							(mg/100g of food)	mg choline moiety/100 g of food						
3	1121	Activia Vanilla Yogurt	1121	Yogurt, 1% to 2% MF	Dairy and Eggs	Dairy	0.8	2.1	7.8	1.6	1.5	1.1	14.1	1121
4	98009	Agave, nectar, all natural	98009	Agave, cooked	Vegetables and vegetable products		0.4	5.2	0.7	0	2.8	0.1	8.8	98009
5	14006	ALCOHOLIC, BEER, LIGHT, 4% ALCOHOL BY VOLUME	14006	Alcoholic beverage, beer, light	Beverages		6.3	5.4	2.5	0	0	0	7.9	14006
6	14096	ALCOHOLIC, DESSERT WINE, SWEET, 18.8% ALCOHOL BY VOLUME	14096	Alcoholic beverage, wine, table, red	Beverages		0.3	4.5	1.1	0	0	0	5.6	14096
7	2	ALCOHOLIC, GIN, 40% ALCOHOL BY VOLUME	2	not analyzed	.		0	0	0	0	0	0	0	2
8	2	ALCOHOLIC, LIQUEUR, COFFEE & CREAM, 17% ALCOHOL BY VOLUME	2	not analyzed	.		0	0	0	0	0	0	0	2
9	2	ALCOHOLIC, LIQUEUR, COFFEE, 26.5% ALCOHOL BY VOLUME	2	not analyzed	.		0	0	0	0	0	0	0	2
10	14003	ALCOHOLIC, REGULAR BEER, 5% ALCOHOL BY VOLUME	14003	Alcoholic beverage, beer, regular	Beverages		8.1	5.7	4.2	0	0	0	9.9	14003
11	2	ALCOHOLIC, RUM, 40% ALCOHOL BY VOLUME	2	not analyzed	.		0	0	0	0	0	0	0	2
12	14096	ALCOHOLIC, TABLE WINE, ALL, 11.5% ALCOHOL BY VOLUME	14096	Alcoholic beverage, wine, table, red	Beverages		0.3	4.5	1.1	0	0	0	5.6	14096
13	14096	ALCOHOLIC, TABLE WINE, RED, 11.5% ALCOHOL BY VOLUME	14096	Alcoholic beverage, wine, table, red	Beverages		0.3	4.5	1.1	0	0	0	5.6	14096

Column A and C are Assigned NBD and refer to the assigned Nutrient Database (NDB) numbers that have been given to the particular food that was recorded. The NDB number assigned to each food is a five-digit numerical code that is used in the USDA Nutrient Database for Standard Reference (SR). Foods included in the USDA choline database that did not have a corresponding SR were given NDB numbers beginning with "98". These assigned NDB numbers were included in the Alberta Database. Foods that were added to the Alberta Database and did not have a NDB

number were given a five or six digit assigned NDB beginning with “888”. A “0” for NDB is assigned if data is missing from that food that is listed. Assigned NDB of “1” indicates that no choline is present in the food item that was entered. When a “1” occurs in the Assigned NDB column then values of “0” will be seen in the columns containing the choline content information (columns G to M). A “2” for NDB indicates that the food item has not been analyzed for choline content and the choline content cannot be accurately estimated. In this case “0” will be seen in the columns containing the choline content information.

The NDB number is used to link the choline content of the food entered in the P column (Item Name) in the Timepoint (A-E) Worksheets. The Assigned NDB column is required to estimate choline intake as it uses a value-lookup (v-lookup) Excel formula to fill in the choline values for each of the foods entered in the Item Name column. The v-lookup formula links the appropriate assigned NDB in the Food Records worksheet to the appropriate assigned NDB number in the Food Processor worksheet. The choline information from that specific food is then transferred from the Food Processor worksheet back to the Food Records worksheet with the help of a conversion factor, which is calculated to fit the quantity of the food recorded.

Food Processor Description (Column B) contains a list of almost every food that was encountered while analyzing the 24-hour dietary recalls of the participants from the study in Chapter 4. The food description is used by Food Processor and includes a variety of similar foods.

USDA Description (Column D) contains the food item that matches the description that is listed in the USDA Database and can be applied to foods with similar wording in Food Processor Description column.

Food Category (Column E) categorizes a list of foods in the Assigned NDB column into food groups.

Dairy or Eggs (Column F) divides the Food Category column, into either “Dairy”, “Eggs” or blank. The Dairy or Eggs column uses another v-lookup formula to first identify if the food listed in the Assigned NBD column falls under the “dairy and eggs” food group, and then identifies if the food is either a dairy product or an egg product. These food groups are the major sources of dietary choline.

The choline and betaine content for each food is listed in columns G to M as betaine, free choline (Free Cho), glycerophosphocholine (GPC), phosphocholine (Pch), phosphatidylcholine (Ptdcho), sphingomyelin (SM) and total choline (TC).

b) Timepoint (A-E) Worksheets

This worksheet contains both the Choline Database and 24-hour dietary recalls that were entered into Food Processor Structure Query Language (SQL). Food Processor SQL is a nutrient analysis software program that has been used in the study from Chapter 4 to analyze macro and micronutrient intake of participants. These worksheets are arranged by visit (LC, HFC, HPCM, HPCS). These worksheets are used to enter data in order to estimate choline intake. *Figure A.2* is a fragment of a Timepoint worksheet, with participant data that must be entered highlighted in red columns and the estimated choline content for each food item consumed calculated in columns in green.

Figure A.2. Timepoint Worksheets: Participant Data (solid) and Choline Content Data (dotted)

O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF
Individual Food Data																	
Participant ID	Food Item Name	Quantity	Measurement	Quantity (g)	Time	Day	Assigned NDB	Food Category	Dairy or Eggs	Factor	Betaine	Free Cho	GPC	Pch	Ptdcho	SM	Total Choline
X	coffee, brewed	250.0	Gram	250	Breakfast	1	14209	Beverages	0	2.50	0.25	4.75	1.75	0.00	0.00	0.00	6.50
X	sugar, white, granulated	5.0	Gram	5	Breakfast	1	1	.	0	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
X	granola bar, peanut, Sweet & Salty Nut	35.0	Gram	35	Morning sn	1	19015	Snacks	0	0.35	2.42	1.47	1.37	0.11	4.80	0.00	7.74
X	apple, gala, fresh	55.0	Gram	55	Morning sn	1	9003	Fruits and fruit products	0	0.55	0.06	0.17	0.00	0.00	1.71	0.00	1.87
X	peanuts, dry roasted	20.0	Gram	20	Morning sn	1	16087	Legumes and Legume Products	0	0.20	0.12	3.52	0.26	0.36	6.36	0.00	10.50
X	chicken nuggets, white meat, frozen	120.0	Gram	120	Lunch	1	98041	Fast foods	0	1.20	27.36	6.60	2.52	1.80	37.80	5.88	54.60
X	mustard, honey	10.0	Gram	10	Lunch	1	2	.	0	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Participant ID (Column O) identifies the code assigned to participants in the study of Chapter 4.

Item Name (Column P) contains the food data is entered. In order for the database to calculate choline content of the appropriate food, the wording must correspond to the wording according to Food Processor.

Quantity (Column Q) refers to the amount of food that was consumed.

Measurement (Column R) is correlated with Quantity and which identifies what classification the Quantity column was measured in.

Quantity (g) (Column S) lists the weight of the individual foods that have been entered. In Food Processor, this information is automatically generated based on the quantity given so this is easily imported into the worksheet. It very important that the gram measurement of the food be correct to calculate choline content from the foods listed in the Choline Database worksheet.

Time (Column T) refers to the time point of the meal from the 24-hour dietary recall.

Day (Column U) is the visit day of in which the data was collected.

Assigned NDB (Column V) refers to the assigned NDB used in the Choline Database Worksheet (explained above). The Assigned NDB column is the most important aspect when estimating choline intake as it uses a value-lookup (v-lookup) Excel formula to fill in the choline values for each of the foods entered in the Item Name column. The v-lookup formula links the appropriate assigned NDB in the Choline Database to the appropriate assigned NDB number in the Timepoint worksheets. The choline information from that specific food is then calculated using a conversion factor, which is calculated to fit the quantity of the food recorded.

Food Category (Column W) categorizes the food listed in the Item Name column into food groups. This also uses a v-lookup formula to assign the appropriate food group to the assigned NDB number that was given.

Dairy or Eggs (Column X) clasified the Food Category column, as either “Dairy”, “Eggs” or blank. It uses another v-lookup formula to first identify if the food listed in the Item Name column falls under the “dairy and eggs” food group, and then identifies if the food is either a dairy product or an egg product.

Factor (Column Y) is the conversion factor that was used to convert the choline information from the Choline Database. For each food in the Choline Database worksheet, the choline content is listed as mg of choline per 100 g of food. The conversion factor is needed to translate the choline content information from the choline database to the accurate amount of food that was listed by each participant.

The food item is entered in the Item Name column, in addition to the amount of food in grams, and then the conversion factor in the Factor column converts the choline content information from Choline Database worksheet to the choline content information appropriate to the foods recorded in the 24-hour dietary recalls. The choline content that has been estimated for each food recorded in the Item Name column is listed in columns Z to AF including betaine, free choline (Free Cho), glycerophosphocholine (GPC), phosphocholine (Pch), phosphatidylcholine (Ptdcho), sphingomyelin (SM) and total choline (TC).

c) Choline Summary

Summary of the choline intake for each participant and each visit (Columns AH-AP). *Figure A.3* is a fragment of the summary of choline intake by participant.

Figure A.3. Choline Intake by participant

AH	AI	AJ	AK	AL	AM	AN	AO	AP
Daily Participant Data								
Particpant ID	Betaine	Free Cho	GPC	Pch	Ptdcho	SM	Total Choline	Meeting AI (<550 mg/day)
	(mg/g per day)	mg choline n	mg choline n	mg choline n	mg choline n	mg choline n	(mg/g per day)	
X-1	44.51	38.50	26.86	8.44	88.70	14.77	177.28	FALSE
X-2	149.18	62.04	89.38	16.12	68.02	15.97	251.53	FALSE
X-3	52.30	51.09	21.38	4.02	74.22	8.73	159.44	FALSE
X-4	65.63	42.85	11.67	0.57	64.55	2.00	121.63	FALSE
Y-1	300.97	82.47	50.58	19.68	185.78	45.00	383.51	FALSE
Y-2	71.75	28.40	16.77	2.46	184.17	21.10	252.91	FALSE
Y-3	113.77	75.59	44.35	15.61	193.75	39.82	369.12	FALSE
Y-4	563.23	249.89	26.79	11.88	434.13	47.50	770.20	TRUE

Meeting AI (Column AP) lists whether each participant met the Adequate Intake (AI) values for choline (550 mg/d). An Excel formula is used to indicate whether the participant is below the AI (listed as FALSE) or above the AI (listed as TRUE).